Isolation and Characterization of *Enterobacter* species from Environmental and Clinical samples in Adama, Ethiopia

Sifan Berhanu Gemule

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

December, 2024

Adama, Ethiopia

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Advisor: Ketema Tafess Tulu (PhD)

Co-advisor: Bayisa Chala (PhD)

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Declaration

I declare that this master thesis entitled "**Isolation and Characterization of *Enterobacter* species from Environmental and Clinical samples in Adama, Ethiopia**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Sifan Berhanu		30-Nov-2024
Name of Student	Signature	Date

Recommendation of Advisors

We the advisor and co-advisor of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Isolation and Characterization of *Enterobacter* species from Environmental and Clinical samples in Adama, Ethiopia**" prepared under our guidance by Sifan Berhanu submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Ketema Tafess Tulu (PhD)		30-Nov-2024
Major Advisor	Signature	Date
Bayisa Chala (PhD)		6-Dec-24
Co-advisor	Signature	Date

Approval Page

We, the advisors of the thesis entitled "**Isolation and Characterization of *Enterobacter* species from Environmental and Clinical samples in Adama, Ethiopia**" and developed by Sifan Berhanu, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Ketema Tafess Tulu (PhD)		30-Nov-2024
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Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Sifan Berhanu have read and evaluated the thesis entitled "**Isolation and Characterization of *Enterobacter* species from Environmental and Clinical samples in Adama, Ethiopia**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Final approval and acceptance of the thesis proposal is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

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Table of Contents

List of Acronyms/Abbreviations

ABIS	Advanced Bacterial Identification Software
AME	Aminoglycoside Modifying Enzymes
ASTU	Adama Science and Technology University
ATP	Adenosine triphosphate
CAP	Community Acquired Pneumonia
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
COVID-19	Corona Virus Disease of 2019
CV	Crystal violet
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleoside triphosphate
DOS	2-deoxystreptamine
EPS	Extracellular Polymeric Substances
ESBL	Extended-Spectrum Beta-Lactamases
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*
HCCA	-cyano-4-hydroxycimmonic acid
ICU	Intensive Care Unit
IGS	Inter Genomic Spacer
IPS	Institute of Pharmaceutical Science
LB	Luria Bertani
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

MDR	Multidrug resistant
MR-VP	Methyl red-Voges Proskauer
NCBI	National Center for Biotechnology Information
NI	Nosocomial Infection
OD	Optical Density
OMP	Outer Membrane Proteins
OMV	Outer Membrane Vesicles
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistant
RND	Resistance Nodulation cell Division
rRNA	Ribosomal ribose nucleic acid
SIM	Sulfur Indole Motility
UV	Ultra Violet
XDR	Extensive Drug Resistant

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Abstract

Enterobacter is emerging as one of the most important bacterial genera due to the increasing prevalence of drug resistance observed in this species. This study aimed to isolate and characterize Enterobacter species from environmental and clinical samples. A total of 65 samples were collected, comprising 40 environmental samples and 25 clinical samples. Bacterial isolates were identified using standard culture methods and characterized through biochemical tests and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Additionally, biofilm formation was assessed using the crystal violet method, and antimicrobial susceptibility was evaluated using the disk diffusion method. Molecular characterization on selected isolates was performed through PCR amplification of the intergenic spacer region and 16S rRNA sequencing. In this study a total of 88 bacterial isolates were identified, of which 41 (46.6%) were identified as Enterobacter spp. based on Gram staining, biochemical tests, and MALDI-TOF MS results, comprising 21 isolates from soil and 20 isolates from clinical samples. Among these, 17 (80%) of the soil isolates and 18 (90%) of the clinical isolates were found to be strong biofilm producers. The soil isolates predominantly exhibited resistance to chloramphenicol and ampicillin, followed by cephalexin. Similarly, the clinical isolates demonstrated high resistance to ampicillin, chloramphenicol, and cephalexin. Notably, the both soil and clinical isolates were found susceptible to cefepime. PCR amplification of the IGS region indicated that two of the isolates (ENV048 and ENV052) share an evolutionary relationship, while the remaining four samples represent distinct strains, suggesting genetic divergence. The 16S rRNA sequencing of isolate ENV053 revealed 98% similarity to E. asburiae. Overall, we demonstrated that Enterobacter spp. are prevalent in both environmental and clinical samples, displaying significant biofilm formation and resistance to available therapeutic options. This warrants close monitoring to prevent the spread of drug resistance.

Keywords: Antimicrobial, Biofilm, *Enterobacter* species, IGS, MALDI-TOF MS, Molecular characterization, PCR

CHAPTER 1

1. INTRODUCTION

1.1 Background of the Study

Enterobacter spp. have recently emerged as significant nosocomial pathogens, posing serious threats in hospital settings. Particularly in the past decade, infectious episodes due to antibiotic resistance of *Enterobacter* spp. have been observed in various regions in the world. It is Classified as an ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) by WHO which is the leading cause of nosocomial infections throughout the world (Shlaes & Bradford, 2018). Notably, resistance to third-generation cephalosporins among these pathogens is a critical concern, as it is linked to increased mortality and morbidity from infections. Infections acquired in hospitals, especially those caused by multidrug-resistant strains of *Enterobacter*, can lead to severe outcomes. *Enterobacter* spp. are responsible for various hospital-acquired infections, including bloodstream infections (BSIs), respiratory infections, urinary tract infections (UTIs), ophthalmic infections, central nervous system infections, and skin and soft tissue infections. Among these, BSIs are the most invasive and severe. *Enterobacter* spp. are ranked as the seventh most common infectious agents in nosocomial pneumonia and the ninth in bloodstream infections (Siddhardha *et al.*, 2020).

Enterobacter is a fermentative gram-negative bacterium, which belongs to the *Enterobacteriaceae* family. This genus was described for the first time by Hormaeche and Edwards in 1960 (Janda & Abbott, 2014). The ability of the genus *Enterobacter* spp. to survive in a wide range of environmental conditions leads to the outbreak of various infections in medical settings. These pathogens are mostly members of the gastrointestinal microbiota and respiratory tract of humans and animals (L'Haridon Stephane & Roussel, 2020). These bacteria are facultative, anaerobic, rod-shaped, and motile by peritrichous flagella, and their glucose fermentation results in acid and gas production. Most *Enterobacter* strains have been reported to have a positive Voges-Proskauer reaction and a negative methyl red test. However, no selective media is available for *Enterobacter* spp. Normally, in clinical setting they are associated with the contaminations caused by intravenous injection fluids, blood products, stethoscope, cotton swabs, and colonized hands of healthcare professionals (dos Santos *et al.*, 2015).

In this genus, classification was described after delineation via DNA-DNA hybridizations. To date, 22 species have been detected in this genus (Davin-Regli *et al*., 2019). The species has been remarked as an opportunistic pathogen involved in a wide array of infections, while the exact mechanism of pathogenicity remains unclear. They could form biofilms and contribute to the secretion of multiple cellular toxins, such as enterotoxins, hemolysins, and pore-forming toxins. Type III secretion system and curli fimbriae may contribute to their pathogenesis as well (Pati *et al*., 2018). The genus *Enterobacter* is associated with a variety of environmental habitats. They are commonly found in water, sewage, soil, plants, or animal feces. They are endophytic or considered as phytopathogens for various plant species. Moreover, *Enterobacter* spp. are also natural commensals of the animal and human gut microbiota. Among these bacteria, only certain subspecies/species have been associated with hospital acquired infections and outbreaks (Singh *et al*., 2018). *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Enterobacter hormaechei* represent the most frequently isolated species described in clinical infections, especially in immunocompromised patients and those hospitalized in an intensive care unit (ICU), due to the adaptation of these species to antimicrobial agents and their behavior as opportunistic pathogens. The wide use of extensive broad-spectrum antibiotics has stimulated the spread of resistant clones (Akbari *et al.*, 2016).

These pathogens are frequently associated with a multidrug resistance (MDR) phenotype, mainly due to their adaptation to the hospital environment and their ability to easily acquire numerous genetic mobile elements containing resistance and virulence genes. These species have an intrinsic resistance to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin due to the expression of a constitutive AmpC β-lactamase. Aminoglycoside and carbapenem resistance have also been reported in this bacterium (Raji *et al.*, 2015). Moreover, the production of extended-spectrum β-lactamases has been reported in these bacteria, which make their treatment difficult. The frequency of the clinical isolates of *Enterobacter* spp. producing extended-spectrum beta-lactamases (ESBL) highly varies in different countries. For instance, the rate has been estimated at 52.1% in Nigeria, 9% in Iran, 14.93% in Pakistan, 37.9% in France, 21.5 % in Taiwan, 6.7% in Barcelona, 7.6% in the Netherlands, and 17.7% in Algeria (Farsiani *et al.*, 2019). Based on a study by Alemayehu *et al*., (2023) prevalence of carbapenemase-producing Enterobacteriaceae in Ethiopia is 5.44% (95% CI: 3.97–6.92). This prevalence is highest in Central Ethiopia at 6.45% (95%

CI: 3.88–9.02) and lowest in the Southern Nations and Nationalities People Region at 1.65% (95% CI: 0.66–2.65).

Molecular techniques have emerged as crucial methods for analyzing microorganisms in a wide range of biological materials, transforming the landscape of microbiology. The 16S rRNA gene is a fundamental element in the study of bacterial phylogenetics, species delineation, and microbiome research. As a key component of the small subunit of prokaryotic ribosomes, it plays a critical role in protein synthesis in both bacteria and archaea. The 16S rRNA gene sequences are the most widely used housekeeping genetic markers for studying bacterial phylogeny and taxonomy for several reasons. First, this gene is found in nearly all bacteria, often occurring as part of a multigene family or in operons. Second, the function of the 16S rRNA gene has remained stable over time, indicating that random sequence changes can serve as a reliable measure of evolutionary time. Lastly, the 16S rRNA gene, which is approximately 1,500 base pairs long, provides sufficient length for effective bioinformatics analysis (Janda & Abbott, 2007). One of the most significant features of the 16S rRNA gene is its high degree of conservation across different species, making it an invaluable molecular marker for phylogenetic studies. This conservation allows researchers to analyze evolutionary relationships among a wide range of microorganisms, from well-studied pathogens to unculturable environmental species. By comparing sequences of the 16S rRNA gene, scientists can effectively delineate species, classify bacterial taxa, and explore the genetic diversity within microbial communities (Hassler *et al*., 2022).

To the best of the researcher knowledge, studying the isolation and characterization of *Enterobacter* spp. in soil and clinical samples in Adama has not been undertaken before. Thus, this study aims to isolate and characterize *Enterobacter* spp. from environmental and clinical samples, as well as to assess the antibiotic resistance patterns, and biofilm formation capacities of *Enterobacter* spp. collected in Adama.

1.2 Statement of the Problem

Enterobacter spp. is an opportunistic pathogen that can cause a wide range of infections, particularly in healthcare settings such as hospitals and long-term care facilities. It is known for its ability to survive and persist on various surfaces and equipment, making it a frequent cause of healthcare-associated infections, including pneumonia, bloodstream infections, urinary tract infections, and wound infections. It can survive in the environment and can be

transmitted through direct contact with contaminated surfaces, healthcare personnel, or medical equipment. Understanding these sources is crucial for implementing effective infection control strategies and preventing the transmission of *Enterobacter* spp. infections.

One of the major concerns associated with *Enterobacter* spp. is its increasing resistance to multiple antimicrobial agents, including antibiotics commonly used in clinical practice. This multidrug resistance poses significant challenges in the treatment of *Enterobacter* spp. infections and necessitates the use of limited therapeutic options, often leading to prolonged hospital stays, increased healthcare costs, and higher mortality rates. Despite the growing antibiotic resistance of *Enterobacter* spp., much of the existing literature primarily addresses clinical isolates, leaving a gap in knowledge regarding the diversity and antibiotic resistance of *Enterobacter* strains from environmental sources, such as soil. Second, there is a notable lack of comprehensive research on their isolation, characterization, and antibiotic resistance profiles, particularly in Ethiopia.

Due to its ability to rapidly acquire and transfer resistance genes, *Enterobacter* spp. has emerged as a global public health concern. This study will be aimed at isolation of *Enterobacter* spp. both from environmental and clinical samples and phenotypical and genotypical characterization of the bacterial isolates. Efforts to isolate and characterize *Enterobacter* spp. both from environmental and clinical samples are vital for implementing effective infection control measures, developing new treatment strategies, and preventing the further spread of this pathogen.

1.3. Scope of the Study

This study aimed to isolate and characterize *Enterobacter* spp. from environmental and clinical samples. The study was conducted at Adama Science and Technology University, IPS laboratory. MALDI-TOF was conducted at South Korea, Wudassie diagnostic center, and Sebeta Animal Health Institute. 16S rRNA sequencing was done at MRC-ET diagnostic center. In the study *Enterobacter* spp. was isolated both from environmental (soil) and clinical (from patients) samples by strictly following standard protocol. After the isolated bacteria was cultured, phenotypical and genotypical characterization was undertaken. The isolated bacteria were characterized by different biochemical tests, biofilm assays, MALDI-TOF MS, Antimicrobial susceptibility test, and 16S rRNA sequencing. This study did not address the epidemiology of *Enterobacter* spp. in Adama.

1.4. Significance of the Study

Since the research study addresses the characterization of *Enterobacter* spp. and its antibiotic resistance, it holds great significance due to several reasons. One is that, *Enterobacter* spp. is a significant opportunistic pathogen that can cause a wide range of infections, particularly in healthcare settings. It is associated with morbidity and mortality rates, especially in immunocompromised individuals. Understanding the growth parameters and virulence factors of *Enterobacter* spp. is crucial for improving clinical management, developing effective treatment strategies, and reducing the burden of *Enterobacter* spp. infections. The other is that, *Enterobacter* spp. has become known for its ability to develop resistance to multiple antimicrobial agents. Studying *Enterobacter* spp. is important for monitoring antimicrobial resistance patterns and identifying emerging resistance mechanisms. This knowledge is crucial for developing alternative treatment options. It also contributes for science community by advancing scientific knowledge in the field of diseases that causes infection.

1.5. Objectives of the study

1.5.1. General objective

- The general objective of this study is to isolate and characterize *Enterobacter* species from environmental and clinical samples from Adama, Ethiopia.

1.5.2 Specific objectives

- To isolate *Enterobacter* spp. both from environmental and clinical samples from Adama, Ethiopia
- To characterize the bacterial isolates using spectrums of phenotypic methods and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.
- To determine antibiotic susceptibility patterns of the bacterial isolates
- To determine the degree of biofilm formation of the bacterial isolates
- To characterize the bacterial isolates genotypically using 16S rRNA sequencing

CHAPTER 2

2. LITERATURE REVIEW

2.1. *Enterobacter* species: Microbiology and Pathogenesis

2.1.1. Taxonomy of the genus

The *Enterobacter* genus belongs to the family *Enterobacteriaceae*, which is a large family of Gram-negative bacteria. The genus encompasses a diverse group of bacteria that are commonly found in various environments, including soil, water, plants, and the intestinal tracts of humans and animals. Many species within this genus are opportunistic pathogens and can cause infections in humans, particularly in individuals with weakened immune systems or underlying health conditions (Mohseni Afshar *et al.*, 2021). To date, 22 species have been found in the genus *Enterobacter: E. aerogenes, E. amnigenus, E. arachidis, E. asburiae, E. carcinogenus, E. cloacae, E. cowanii, E. dissolvans, E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli, E. taylorae,* and *E. turicensis.* Among these species, seven are grouped within the *Enterobacter cloacae* complex group: *E. cloacae, E. asburiae, E. hormaechei, E. kobei, E. ludwigii, E. mori,* and *E. nimipressuralis*. This nomenclature is based on the sharing of phenotypic and especially genotypic characteristics, determined by whole-genome DNA-DNA hybridizations (Davin-Regli *et al*., 2019). Indeed, *E. cloacae* shares at least 60% similarity in its genome with the other six members of this group. The development of genome sequencing has rapidly modified the phylogeny of the genus, particularly that of the *E. cloacae* complex (Liu *et al.,* 2013). Here is a taxonomy breakdown of the *Enterobacter* genus:

KINGDOM: Bacteria

PHYLUM: Proteobacteria

CLASS: Gammaproteobacteria

ORDER: Enterobacterales

FAMILY: *Enterobacteriaceae*

GENUS: *Enterobacter*

2.1.2. Morphology and structure

The *Enterobacteriaceae* are a large family of bacteria, including many of the more familiar pathogens, such as *Salmonella, Shigella* and *Escherichia coli*. Members of the *Enterobacteriaceae* are bacilli (rod-shaped), facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. They are typically 1-5 µm in length and they have Gram-negative stains. Most have many flagella used to move about, but a few genera are non-motile. They do not form spores. Most members of *Enterobacteriaceae* have fimbriae involved in the adhesion of the bacterial cells to their hosts (Louisiana Office of Public Health, 2018).

As shown in Figure 1 *Enterobacter* spp. typically appear as straight or slightly curved rods, giving them a rod-shaped morphology. The length and width of the cells may vary depending on the species and growth conditions generally being 1-5 µm in length and 0.5-1.0 µm in width when they grow on a solid agar media. They have flagella which is used for motility. They can be cultured on various types of media commonly used for bacterial isolation and identification such as Nutrient agar, MacConkey Agar, Eosin Methylene Blue (EMB) Agar, and Blood agar (Nyenje *et al.*, 2014).

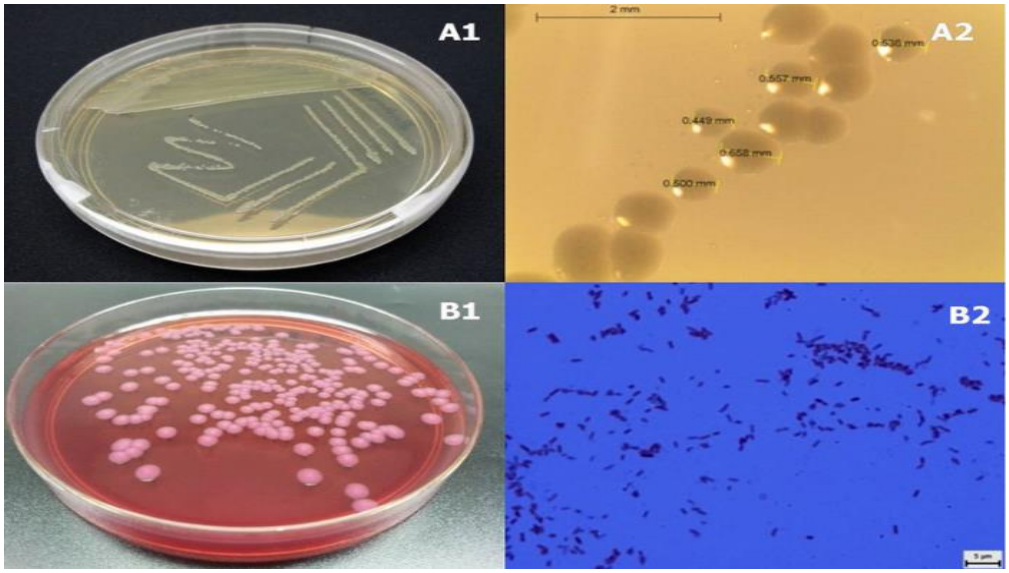

Figure 1. *Enterobacter cloacae* colony characteristics (Cosmas *et al*., 2016).
Key: A: Nutrient agar (A1: Colony form in nutrient agar; A2: Colony view under stereo microscope). B: MacConkey agar (B1: Colony form in MacConkey agar, B2: Microscope view showed Gram-negative and rod shape bacteria, 1.4 um at 100x magnification)

2.1.3. Mechanisms of pathogenicity

Little is known about the pathogenicity and virulence factors of *Enterobacter* spp. due to the scarcity of studies in this area. *Enterobacter* spp. can exhibit pathogenicity through various mechanisms that contribute to their ability to cause infections. *Enterobacter* spp. possess adhesins, such as pili and fimbriae, that enable them to adhere to host tissues and surfaces. Adherence is a crucial step for colonization and establishment of infection in various sites, including the urinary tract, respiratory system, and surgical wounds. For instance, the fimH and mrkD genes, encoding adhesins of type 1 and type 3 fimbriae, and ycfM are detected in a study, and they play a key role in bacterial adhesion and in biofilm formation, which are important aspects of bacterial virulence (El Fertas-Aissani *et al.,* 2013). In addition to Adhesion, flagella possess several other functions like biofilm formation, protein export, and facilitating motility. This species can form biofilms, which are complex communities of bacteria encased in a matrix of extracellular polymeric substances. Biofilms provide protection against host immune responses and antimicrobial agents, making it more challenging to treat infections. Biofilms are commonly associated with medical device-related infections, such as catheter-associated urinary tract infections (Haiko & Westerlund-Wikström, 2016).

Enterobacter spp. can produce a range of virulence factors that contribute to their pathogenicity. Certain *Enterobacter* species can produce a polysaccharide capsule that helps protect the bacteria from host immune defenses and phagocytosis. Some possesses different endotoxins. LPS is a component of the outer membrane of Gram-negative bacteria, including *Enterobacter* spp. LPS can induce an inflammatory response in the host, contributing to the pathogenesis of infection. They can also produce proteases that degrade host proteins, facilitating tissue invasion and immune evasion. Some strains produce hemolysins, which can damage host red blood cells and contribute to tissue damage (Davin-Regli *et al.,* 2019).

In Gram-negative bacteria, the type III secretion system (T3SS) is recognized as a pathogenicity factor. One study showed that 27% of *E. cloacae* isolates from clinical infections possessed this factor. Certain strains of *E. cloacae* possess a T3SS, which is a complex molecular machinery that allows the bacteria to inject effector proteins directly into host cells. These effector proteins can manipulate host cell signaling pathways, interfere with immune responses, and promote bacterial survival and replication (Davin-Regli *et al.,* 2019). *Enterobacter* spp. can also employ quorum sensing, a cell-to-cell communication system, to regulate the expression of virulence factors and coordination of bacterial behavior. Quorum

sensing allows the bacteria to sense and respond to changes in population density, promoting the expression of specific genes required for infection. It is important to note that the pathogenicity mechanisms can vary among different species and strains within the *Enterobacter* genus. Additionally, the specific combination of virulence factors and mechanisms employed by *Enterobacter* spp. may depend on the site of infection and the host environment (Muhammad *et al.,* 2020).

2.2. Biofilm formation

A biofilm is a three-dimensional structure that serves as a battleground for microorganisms. It is typically defined as a stable community of microbes encased in extracellular polymeric substances (EPS). The EPS form a sticky matrix predominantly composed of water channels that facilitate the transport of nutrients and oxygen. Besides providing a protective barrier against the host's immune defenses, such as antibodies and white blood cells, and antibiotics, the EPS acts as a fundamental base for microbial attachment to surfaces. Furthermore, it has been demonstrated to enhance the activity of intercellular signaling molecules, playing a crucial role in communication among the cells within the biofilm (Khatoon *et al*., 2018). Biofilms are characterized by the irreversible adhesion of microbial cells to surfaces, each other, or a combination of both, all embedded within the EPS matrix. A bacterial biofilm can consist of a single species or a diverse mixture of bacteria, fungi, archaea, protozoa, and yeasts, featuring a channel-like structure that regulates the flow of gases, nutrients, and antimicrobials.

Biofilms are prevalent in nature, found on various surfaces such as rocks in streams, mammalian teeth, plant roots, and even within water pipes. They represent one of the most adaptable microbial features in the environment. However, when the microorganisms involved are pathogenic, their ability to form biofilms becomes a critical virulence factor. In fact, most human infections are biofilm-related (Ibrišimović, 2017). These infections are often associated with medical devices, such as knee replacements, catheters, implants, and prosthetic joints, as well as tissue-related conditions like chronic wounds, staphylococcal skin infections, endocarditis, chronic otitis media, and lung infections in cystic fibrosis. Furthermore, biofilms can hinder the effectiveness of antimicrobials and influence immune responses, contributing to antimicrobial resistance and facilitating the development of persistent or chronic infections. Because these biofilms are frequently polymicrobial, they can become exceedingly difficult, if not impossible, to treat effectively (Zhao *et al*., 2023).

Biofilms are generally formed as a natural defense mechanism, allowing bacteria to create a favorable environment, retain essential nutrients, and enhance their chances of survival. While the basic process is similar across different bacterial species, variations can occur. The growth of bacterial biofilms typically results from a combination of physical, chemical, and biological factors. This process is usually divided into three stages: (i) initial attachment (which can be reversible or irreversible), (ii) maturation of microcolonies, and (iii) dispersion or detachment. Attachment is marked by the production of bacterial adhesins that adhere to surfaces, while the maturation of the biofilm is facilitated by cell-cell adhesion mechanisms. During the dispersal stage, enzymes that break down the biofilm matrix are involved. Figure 2 illustrates these key stages of biofilm formation on implantable devices (Zurob *et al*., 2019).

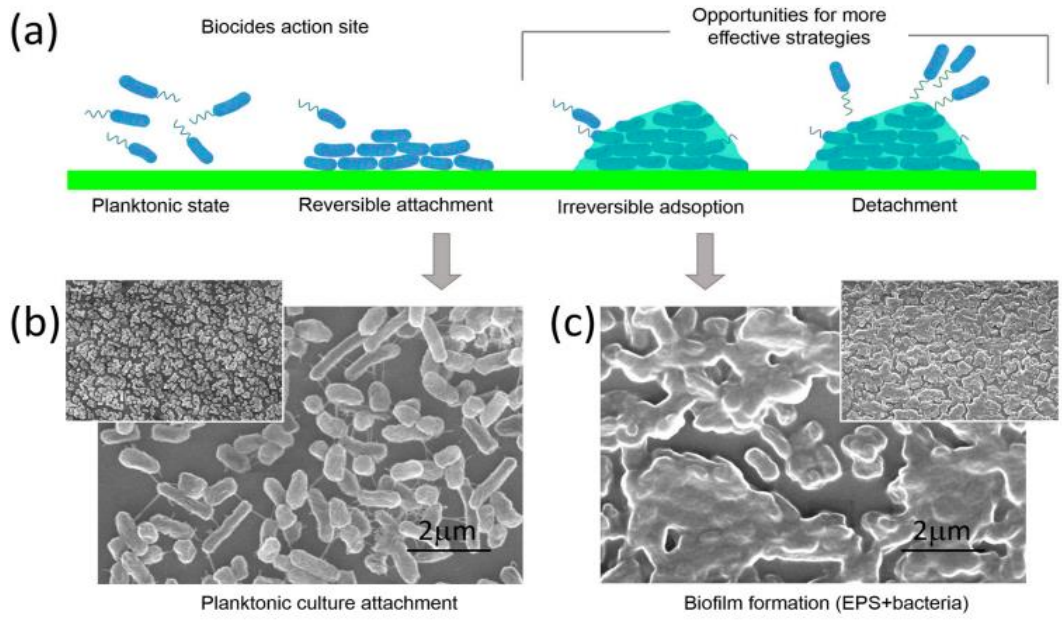

Figure 2. *Enterobacter cloacae* biofilm formation on glass. (a) Illustrative diagram of biofilm formation stages: Planktonic state, reversible attachment, irreversible adsorption, and detachment; (b) Scanning electron image (SEM) of E. cloacae after 24 h incubation (Zurob *et al*., 2019)

2.2.1. Role of biofilms in Antimicrobial Resistance

Biofilms play a complex role in antimicrobial resistance (AMR), significantly increasing resistance levels in bacteria. Bacteria within biofilms can be 10 to 1,000 times more resistant to antibiotics than their planktonic counterparts (Prentice & Webber, 2024). Currently, 60-80% bacterial infections are associated with biofilm formation. Biofilms can protect the pathogen from host immune responses and the antipathogenic effects of antibiotics, thereby improving antibiotic resistance and survivability of the bacteria, in addition to increasing the

difficulty of treating the ensuing disease (Ribeiro *et al*., 2016). Common resistance mechanisms include point mutations, enzyme production, and efflux pumps. These and other factors work together in biofilms to diminish or prevent antibiotic effectiveness, leading to a phenomenon known as recalcitrance. Three key mechanisms of antibiotic resistance in biofilms includes: Resistance at the Biofilm Surface: The sticky, complex structure of the biofilm, composed of exopolysaccharides, DNA, and proteins, hinders antibiotic penetration. Antibiotics may be deactivated at the surface before reaching the bacteria. The second is resistance within biofilm Microenvironments: Deeper within the biofilm, metabolic waste and reduced oxygen create challenging conditions that can alter antibiotic efficacy. For example, low oxygen levels can diminish the effectiveness of antibiotics like tobramycin and ciprofloxacin. The last is that, resistance of bacterial "Persister" Cells: Within the biofilm, some bacteria can enter a dormant "spore-like" state, becoming persister cells that resist antibiotic action without genetic changes. When released from the biofilm or when they begin to divide again, they revert to their original susceptibility. Together, these mechanisms enable bacteria in biofilms to survive high concentrations of antibiotics, complicating treatment efforts (Prentice & Webber, 2024).

2.3. Antibiotic resistance and virulence factors

2.3.1. Overview of antibiotic resistance

The use of antibiotics to treat bacterial infections is a key component of modern medicine but antibiotic resistance, particularly multi-resistance, in the *Enterobacteriaceae* is an increasing global problem, with strains resistant to most (or even all) available antibiotics emerging. Antibiotic resistance poses a significant challenge to public health, representing one of the most prominent threats. The rise and dissemination of MDR bacteria, primarily attributed to the excessive and indiscriminate use of antibiotics, have raised concerns in human medicine. MDR bacterial strains are commonly associated with infections acquired in healthcare settings (nosocomial infections), and their presence has been linked to heightened mortality rates (Nocera *et al.,* 2021).

Enterobacter spp. are the leading causes of nosocomial infections (NIs) with a growing prevalence, and the infections caused by *Enterobacter* spp. with antibiotic resistance have been associated with higher mortality, longer hospitalization, and higher treatment costs compared to the infections caused by other sensitive strains. *Enterobacter* spp., including *E. cloacae* and *E. aerogenes*, are known for their ability to acquire and exhibit antibiotic resistance (Artridge, 2016; Nordmann & Poirel, 2019).

These bacteria can develop resistance through various mechanisms, including the acquisition of resistance genes, mutation of endogenous genes, and the production of enzymes that inactivate antibiotics. β-lactam antibiotics are the most prescribed agents for the treatment of the infections caused by *Enterobacter* spp. This antibiotic group includes penicillin derivatives such as cephalosporins, monobactams, and carbapenems. According to the literature, the most important emerging resistance in *Enterobacter* is resistance to broad spectrum β-lactam antibiotics, which is mostly attributed to the production of β-lactam inhibitors (Partridge, 2012).

2.3.2. Antibiotic Resistance Mechanisms in *Enterobacter*

Resistance mechanisms include hydrolysis or modification of antibiotics, protection, or replacement of antibiotic targets, decreased antibiotic entry and/or increased efflux or modification of the outer membrane. Much of the resistance in *Enterobacter* spp. is due to 'mobile' genes captured from various source species by different mobile genetic elements and transferred to plasmids, which can then move between cells, including of different species and indeed even different genera. Mutations in chromosomal genes are also important in enhancing resistance to certain classes of antibiotics, including some now being employed as last-line antibiotics, such as polymyxins and tigecycline for use against multi drug resistant organisms (Artridge, 2016).

Resistance to β-Lactam Antibiotics

The β-lactam group of antibiotics encompasses penicillin's, cephalosporins, carbapenems, penems and monobactams. They irreversibly bind to the penicillin binding proteins (PBP) required for the final crosslinking step in the synthesis of peptidoglycan for cell wall construction. The most clinically important are the third-generation cephalosporins, 'anti-pseudomonal' β-lactam/β-lactamase inhibitor combinations, and carbapenems (Peirano *et al.,* 2014). The most common reason for resistance to β-lactam antibiotics in the *Enterobacter* spp. is the expression of β-lactamase enzymes (encoded by bla genes) that hydrolyze the β-lactam ring, resulting in inactivation. Many different types of β-lactamases can confer resistance to each of the most clinically-important β-lactam types and a single amino acid difference may affect the phenotype conferred (Shoma *et al.*, 2014).

Resistance to Aminoglycosides

Aminoglycosides constitute a complex family of compounds that have an aminocyclitol nucleus (e.g., 2-deoxystreptamine; DOS) linked to amino sugars through glycosidic bonds.

The most clinically relevant, amikacin (AMK), gentamicin (GEN) and tobramycin (TOB) are 4,6-disubstituted. Most aminoglycoside antibiotics bind to the aminoacyl-tRNA recognition site (A-site), the decoding center on the 16S rRNA of the ribosome, interfering with protein synthesis. Resistance to aminoglycosides in *Enterobacter* spp. is commonly due to mobile genes encoding aminoglycoside modifying enzymes (AME) that modify the DOS nucleus or the sugar moieties and inactivate the aminoglycoside. AME either acetylate -NH2 groups using acetyl co-enzyme A, adenylylate - OH groups by transferring an AMP group from ATP or catalyze ATP-dependent phosphorylation of an -OH group (Jacoby *et al.,* 2014).

Resistance to Quinolones

Quinolones and fluoroquinolones target DNA gyrase and topoisomerase IV enzymes, which modulate the topological state of DNA and have essential roles in many processes involving nucleic acids. DNA gyrase can introduce negative supercoils, while topoisomerase IV is mainly involved in removing knots in DNA. DNA gyrase is a tetramer of two GyrA plus two GyrB subunits while topoisomerase IV is similarly a tetramer of two ParC plus two ParE subunits. Both enzymes introduce double-stranded breaks in the DNA, followed by re-ligation. Quinolones bind to the cleavage-ligation active site and intercalate into the DNA, blocking ligation and enhancing DNA fragmentation, as well as generally impairing DNA gyrase and topoisomerase IV function. Mutations at certain positions in DNA gyrase and/or topoisomerase IV contribute to fluoroquinolone resistance (Wang *et al.*, 2010).

Resistance to Colistin/Polymyxin

Polymyxins, cyclic cationic decapeptides bound to a fatty acid chain, were originally discovered in the 1940s. They target the negatively-charged lipid moiety of lipopolysaccharide (LPS) of the outer membrane, displacing divalent cations (Ca2þ, Mg2þ) that stabilize the LPS. This disrupts the integrity of the outer membrane, causing leakage of intracellular contents and cell death. Routine use of polymyxins was discontinued in the 1980s due to common and serious side effects (nephrotoxicity, neurotoxicity), but the need to find ways of treating multidrug resistant Gram-negative infections has led to a recent revival in their use. Polymyxins B and E (colistin), which differ by a single amino acid, are used clinically, with colistin being the most used in recent years (Falagas *et al.*, 2010). In *Enterobacter* spp., polymyxin resistance can arise by a variety of LPS modifications, e.g., addition of 4-amino-4-deoxy-l-arabinose (L-Ara4N) to the phosphate group of Lipid A, which reduces the negative charges available for interactions. L-Ara4N is synthesized and

added to LPS by proteins encoded by the arn (for AraN synthesis) operon, previously called the pmrHFIJKLM (for polymyxin resistance) or pbg operon (Olaitan *et al.*, 2014).

2.4. *Enterobacter* Infections

Enterobacter spp. are associated with a wide variety of clinical infections such as lower respiratory tract infections, urinary tract infections, bacteremia, skin and soft tissue infections, endocarditis, central nervous system infections, bone and joint infections, and gastrointestinal tract infections. Infections by *Enterobacter* spp. are mostly similar to those by other facultative Gram-negative bacilli. Recently, there has been a notable rise in resistance to third-generation cephalosporins among *Enterobacter* spp. This multidrug resistance poses a serious challenge in treating infections and is associated with high rates of morbidity and mortality (Siddhardha *et al*., 2020)

***Enterobacter* bacteremia:** Bacteremia refers to the presence of bacteria in the bloodstream. It can occur transiently or can lead to severe infections and sepsis. Species associated with *Enterobacter* bacteremia are *E. aerogenes, E. agglomerans, E. cloacae, E. asburiae, E. sakazakii, E. amnigenus, E. gergoviae,* and *E. hormaechei*. Bacteremia occurs in debilitated patients like those who had recently hospitalized, or received corticosteroid therapy, or previously received antibiotics or admitted to ICU. The mortality rate associated with *Enterobacter* bacteremia was 20 and 24% at 14 and 28 days after the diagnosis of bacteremia (Kang *et al*., 2004).

Lower Respiratory Tract Infections (LRTI): Like *Enterobacter* spp. are common cause of bacteremia, most of these species are implicated in LRTIs. They involved in lung abscess, pneumonia, emphysema, asymptomatic colonization in respiratory secretions, and purulent bronchitis. These species surpassed *Klebsiella* spp. and known as the third most cause of nosocomial respiratory tract infections in the USA. It is recognized as a cause of community-acquired pneumonia. In last decades, prevalence of lower respiratory tract infections by *Enterobacter* spp. increased continuously (John *et al*., 1982).

Urinary Tract Infections (UTI): UTIs are infections that can affect any part of the urinary system, including the kidneys, bladder, ureters, and urethra. *E. cloacae* are one of the chief pathogens involved in UTI. ESBL producing *Enterobacter* spp. are major in hospital-acquired urinary tract pathogens. One of the effective antibiotics against ESBL producing strains were carbapenems but the expression of carbapenemase provided resistance to all beta-lactam drugs (Xu & He, 2019).

2.5. Molecular characterization

Molecular techniques have become essential tools for the analysis of microorganisms in various biological substances, revolutionizing the field of microbiology. These advanced methods allow for the screening of a wide array of microbial agents in a single test, providing a comprehensive and efficient approach to microbial identification and characterization. As molecular techniques continue to evolve, their applications are expanding across multiple domains, including clinical diagnostics, environmental monitoring, and food safety. The range of molecular techniques currently in use includes polymerase chain reaction (PCR), next-generation sequencing (NGS), and various hybridization methods, each designed to facilitate rapid differentiation of species and precise strain identification. These methods are particularly valuable for determining strain relatedness of the samples, enabling healthcare professionals and researchers to track outbreaks, understand transmission dynamics, and develop targeted interventions (Babalola, 2003).

2.5.1. 16S-23S rRNA intergenic spacer region

The 16S-23S rRNA intergenic spacer (IGS) region is a variable segment of DNA located between the 16S and 23S ribosomal RNA genes in bacterial genomes. Its considerable variability in length and sequence among different species makes it a valuable target for phylogenetic analysis. While parts of the IGS region may be conserved across different species, other segments can exhibit high variability. This combination allows for both the identification of related organisms and the differentiation of closely related species. The IGS region aids in species identification by allowing researchers to distinguish closely related bacterial species, which is particularly useful in environmental microbiology and clinical diagnostics (Pecchia et al., 1998). Additionally, sequence data from the IGS region can be utilized to construct phylogenetic trees, revealing evolutionary relationships, and providing insights into genetic adaptations. As a molecular marker, the IGS region contributes to understanding bacterial diversity and can be analyzed alongside other genetic markers to enhance comparative genomics studies. Overall, the 16S-23S rRNA IGS region is instrumental in elucidating the complexity of bacterial evolution and diversity (Poaceae, 2017; Mekha *et al*., 2010) .

2.5.2. 16S rRNA sequence

The 16S rRNA gene is used extensively in bacterial phylogenetics, in species delineation, and now widely in microbiome studies. It is a component of the small subunit of prokaryotic ribosomes, crucial for protein synthesis in bacteria and archaea. It plays a vital role in the

assembly and functioning of the ribosome, facilitating the binding of mRNA and tRNA during translation. The 16S rRNA gene is highly conserved across different species, making it an essential molecular marker for phylogenetic analysis and bacterial classification. Researchers often use the 16S rRNA gene for identifying and differentiating bacterial species due to its sequence variability among different taxa (Hassler *et al*., 2022).

In the 1977s, a new standard for identifying bacteria began to be developed. Extensive use of the 16S rRNA gene in phylogenetics was first pioneered by Carl Woese to delineate the previously undescribed taxonomic lineage - Archaea. In the laboratories of Woese and others, it was shown that phylogenetic relationships of bacteria, and, indeed, all life-forms, could be determined by comparing a stable part of the genetic code. Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes. The part of the DNA now most used for taxonomic purposes for bacteria is the 16S rRNA gene (Iii, 2004).

The 16S rRNA gene sequence is approximately 1,550 base pairs long and comprises both variable and conserved regions. Its length and interspecific polymorphisms enable effective differentiation among species, providing statistically valid measurements. Universal primers are typically selected to match the conserved regions at the beginning of the gene and either at the 540-bp region or the end of the sequence, while the variable region in between is utilized for comparative taxonomy. Commonly, sequences of 500 to 1,500 bp are analyzed, though database entries can vary in length. Comparing 16S rRNA gene sequences facilitates differentiation at the genus level across major bacterial phyla and allows for classification at multiple levels, including species and subspecies (Hassler *et al*., 2022).

2.5.3. Phylogenetic analysis

In phylogenetic analysis, branching diagrams are created to illustrate the evolutionary history and relationships between different species, organisms, or traits (such as genes, proteins, or organs) derived from a common ancestor. These diagrams, known as phylogenetic trees or cladograms, serve as graphical representations that resemble trees, depicting the evolutionary connections among biological taxa based on their physical or genetic characteristics. Phylogenetic analysis is crucial for understanding biological diversity, classifying genetic relationships, and uncovering developmental events that occur throughout evolution (Zou *et al.*, 2024).

A phylogenetic tree is structured with a series of branching points that extend from the last common ancestor (the root) of all operational taxonomic units to the most recent organisms

(the tips). The tree features leaves (or tips), nodes, and branches, where two adjacent nodes (representing taxonomic units) are connected by a single internal branch (Figure 3). In this tree, leaves can represent species, populations, individuals, or genes, and they are connected to nodes via external branches. These branches illustrate the transfer of genetic information across generations, with branch lengths indicating genetic change or divergence. The degree of divergence is typically estimated by the average number of nucleotide substitutions per site. As one analyzes the phylogenetic tree from the root to the tips, each node marks the point where two or more descendant lineages emerge from a common ancestor, with evolution occurring independently in these newly formed lineages (Brinkman, 2001).

Phylogenetic analysis offers a comprehensive understanding of how species evolve through genetic changes. By utilizing phylogenetics, scientists can trace the evolutionary path from present-day organisms back to their ancestral origins and predict potential future genetic divergences. This approach has numerous applications across medical and biological fields, including forensic science, conservation biology, epidemiology, drug discovery and design, prediction of protein structure and function, and gene function prediction (Zou *et al*., 2024).

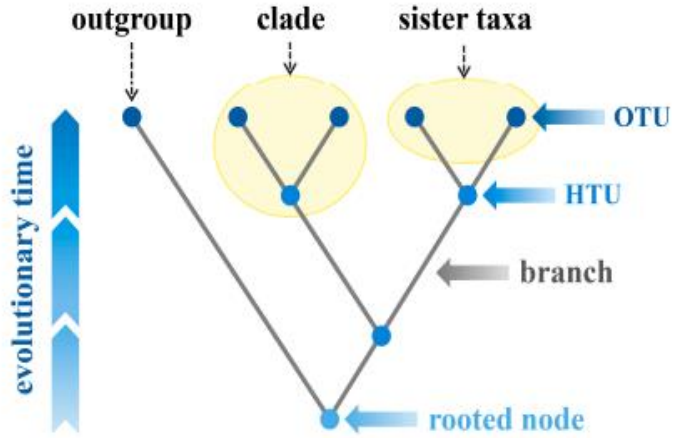

Figure 3. The general structure of a phylogenetic tree. The abbreviations in the figure are as follows: OTU, operational taxonomic unit; HTU, hypothetical taxonomic unit (Zou *et al*., 2024).

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Description of the study area

Soil samples were collected from different non-residential areas in Adama city and inside Adama Science and Technology University from November 9, 2023 to December 30, 2023. Adama is a city located in the East Shewa Zone of the Oromia Region, Ethiopia, located at $8^{0}32'29''N$ 39^{o} 16'08'E and at an elevation of 1,712 meters (Figure 4). It is located between the base of an escarpment to the west, and the Great Rift Valley to the east. The city is characterized by a temperate climate, and the summer season presents some challenges in terms of precise categorization. The average annual temperature in Adama is 20.7 °C | 69.3 °F. Throughout the year, precipitation is scarce in Adama.

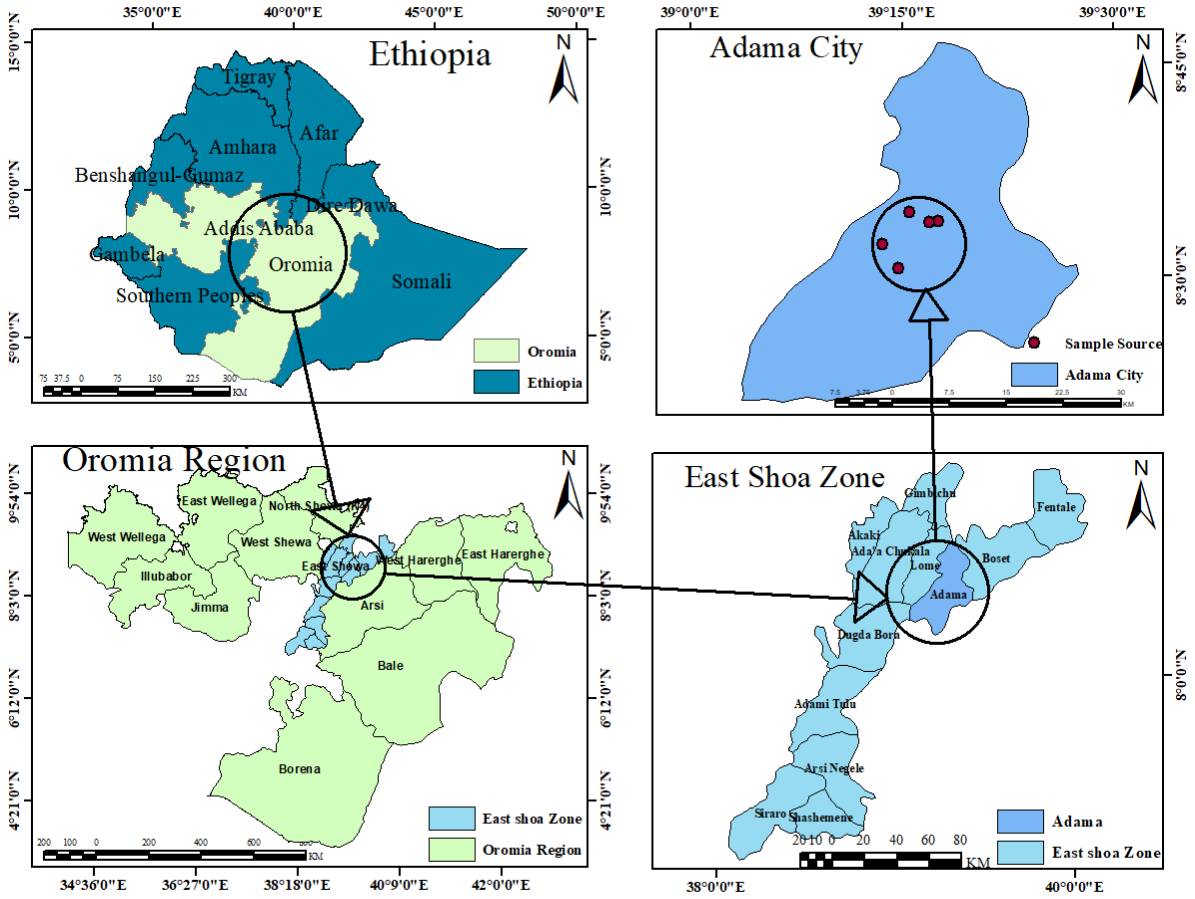

Figure 4. Location map of Adama city and samples collection sites.

Whilst for the clinical sampling, the samples were obtained from clinical samples stored at Adama Public Health Research and Referral Laboratory Center as part of Community-Acquired Pneumonia (CAP) research project. It is found in Oromia Regional State, Adama city, kebele 12 near Adama Comprehensive Specialized Hospital Medical College on street to bole sub-city, 100Km away from Capital city, Finfinnee/Addis Ababa. It serves the community in the catchment area by giving referral laboratory services, Capacity Building, Conducting operational Research, Training and Reagent preparation and distribution.

3.2. Sample Collection and Processing

A total of sixty-five (N=65) samples were collected both from environment and clinic. For the environmental sampling, forty (N=40) soil samples were collected from Adama Science and Technology University (ASTU) compound and Around Adama city at different non-residential areas. A soil sample of 50 gram was collected using a sterile shovel from a depth of 5 cm to ensure that the sample was taken from deep soil. All samples were placed in sterile zip-lock plastic bags, numbered, and stored in an ice box. The samples were transported to the institute of pharmaceutical science (IPS) laboratory on the same day, and stored in a refrigerator at − 2 °C for later use.

For the clinical sample, a total of twenty-five (N=25) sputum samples were collected initially for the isolation and characterization of bacteria associated with Community-Acquired Pneumonia (CAP) (Hamde *et al*., 2024). These samples were stored at -80°C at the Adama Public Health Research and Referral Laboratory Center. We utilized these stored sputum samples for the isolation of *Enterobacter* spp. Each sputum cup containing a specimen was appropriately labeled with a unique sample number. A minimum of 1 mL of sputum from the stored samples was used for microbiological inoculation and bacterial isolation, which was conducted in the microbiology laboratory of the Adama Public Health Research and Referral Laboratory Center. The isolates were then transported to the institute of pharmaceutical sciences (IPS) laboratory for further characterization.

3.3. Isolation of *Enterobacter* spp.

First, the workspace was cleaned by wiping the interior surfaces of the hood with 70% ethanol, and all necessary materials were gathered, including sterile media, inoculating loops, and bacterial samples. After wearing personal protective equipment (PPE) such as gloves and a lab coat, the laminar flow hood was turned on and allowed to run for 10-15 minutes to establish proper airflow. All materials were placed inside the hood, maintaining

a clean area. The inoculating loop was sterilized by flaming it under Bunsen burner until red hot, and then it was allowed to cool briefly. For the environmental samples collected from soils in Adama, a serial dilution (10^{-1} to 10^{-4}) was performed, and the 10^{-2} and 10^{-3} dilutions were used for inoculation. The samples (100 µL) were first cultured on nutrient agar media and incubated for 24 hours at 37°C. To prepare the nutrient agar, 28 grams of the agar powder were measured and added to 1 liter of distilled water. The mixture was then gently heated while being continuously shaken until the agar powder was completely dissolved. The solution was then transferred to an autoclave-safe container and sterilized at 121°C for 20 minutes. After the sterilization, the nutrient agar was poured into sterile petri dishes and left to solidify. The colonies that appeared as smooth, creamy rods with a mucoid texture were considered as presumptive *Enterobacter* spp. colonies. For purification, the MacConkey media was then used (Ababneh *et al*., 2022).

For the clinical samples, blood agar was used for the isolation of bacteria. 50 grams of blood agar base powder was measured and added to 1 liter of distilled water. The mixture was then heated while being stirred continuously until the agar powder was completely dissolved. The agar solution was then sterilized by autoclaving at 121°C for 15 minutes. After cooling the agar solution to 45°C inside water bath, 5% (v/v) of sterile, defibrinated blood (sheep blood), was aseptically added and gently mixed. The blood-agar solution was then poured into sterile petri dishes and allowed to solidify at room temperature. The clinical sample is inoculated onto the surface of a blood agar plate, then incubated at an appropriate temperature, 37°C, for 24 hours to allow the bacteria to grow. The plate is then examined for the presence of bacterial colonies. Distinct colonies are then selected and sub-cultured onto fresh blood agar plates to obtain pure cultures (Regasa *et al*., 2015).

3.4. Identification and characterization of isolated bacteria

3.4.1. Morphological characterization

The bacterial isolates underwent thorough morphological characterization, based on Doijad *et al*., (2016). The colony features, including color, elevation, margin, and surface were meticulously observed, and recorded after 24 hours of growth on a suitable culture medium. Additionally, microscopic examination was conducted to further analyze the isolates' morphological attributes, such as the specific cellular shapes, including rods, cocci, commas, and spirals. This comprehensive approach to morphological analysis provided essential insights into the phenotypic characteristics of the isolated bacteria, contributing to their accurate identification and classification.

3.4.2. Gram staining

Gram staining was performed on the isolated bacteria to differentiate between gram-positive and gram-negative species. Bacterial colonies were smeared on a drop of water on a clean glass slide then were left to air dry and heat fixed. Then the slides were flooded with crystal violate dye for 1 min followed by rinsing under tap water. Then the slides were flooded with iodine for 1 min as a mordant and again rinsed under tap water. After, the slides were rinsed with 70% (v/v) ethanol for 15 sec and then rinsed with tap water. Lastly, the slides were flooded with safranin as a counter stain for 1 min and rinsed with tap water. Next, the slides were allowed to dry and were viewed under microscope x100 with oil immersion (Jesumirhewe *et al*., 2016).

3.4.3. Biochemical characterization

Followed by incubation, the bacterial isolates were used for different biochemical assays including catalase, citrate utilization, indole, sulfur indole motility (SIM), Methyl red - Voges Proskauer (MR-VP), Urea hydrolysis, and Lactose fermentation test for identification as previously described by Jesumirhewe *et al*., (2016), Dimri *et al*., (2020), Mustapha, (2019). These assays are used frequently to identify and differentiate *Enterobacter* Spp. from other gram-negative bacteria. Each of the biochemical test is described below.

3.4.3.1. Catalase test

Catalase test was performed for these newly isolated bacteria samples. It was performed following the methods of Jesumirhewe *et al.*, (2016). Briefly, bacterial isolates were inoculated into nutrient agar. It was incubated at 37^{o}C for 24hrs. After 24hrs, the fresh culture colony was taken and placed on microscopic slide. A 1-2 drops H_2O_2 were added to bacterial isolates placed onto microscopic slide. Bubble gas was formed and considered as positive for catalase enzymes.

3.4.3.2. Citrate utilization test

Citrate test was carried out to test the ability of the bacteria to use citrate as a carbon source. Fresh 18-24 h colonies were streaked on Simmons citrate agar slants (g/l) (Bromothymol blue 0.08; MgSO4 0.2; (NH4)2HPO4 1 and Agar 15) and incubated at 37^{o}C 2- 4 days. Citrate metabolization is indicated by a color change from green to blue.

3.4.3.3. Indole test

Tryptone water medium was inoculated with the test isolate and incubated at 35–37 ˚C for up to 48 h. Kovac's reagent was then added in 0.5 mL volume to the culture and shaken

gently. A red colour in the surface (the alcohol layer) of the medium implied a positive reaction. No-colour change indicates a negative reaction.

3.4.3.4. SIM test

The SIM medium tests for hydrogen sulfide production, indole production, and motility. A sterilized tube containing SIM medium was inoculated with 18-24h old culture and incubated at 37ºC for 24 - 48h. Hydrogen sulfide production was indicated by presence of black precipitate and motility was indicated by growth that spreads out from the initial stab line. Indole production was indicated by the presence or absence of a red ring after 0.5ml of Kovac's reagent was added into test tube.

3.4.3.5. MR-VP test

The MR-VP test is a combination of two separate tests. The MR assay determines if an organism metabolizing glucose utilizes mixed acid fermentation pathway and produces strong acid end products (lactic, acetic, or formic) that are detected by the indicator methyl red. 5 ml MR-VP broth tube was inoculated with the organism and was incubated at 35ºC for 48–72 h. A 2.5 ml of the broth culture was transferred to a fresh tube and inoculated with five drops of methyl red indicator then the presence or absence of color change from yellow to red was recorded.

The VP test detects the production of acetoin by organisms that utilize the butylene glycol fermentation pathway. In the presence of air and potassium hydroxide, acetoin is oxidized to diacetyl, which produces a red-colored complex. A 5 ml MR-VP broth tubes were inoculated with pure bacterial culture and incubated at 35ºC for 18–24 h. A 2.5 ml of the broth cultures were transferred to a fresh tube and inoculated with six drops of α-naphthol followed by three drops of KOH. Then, the tubes were shaken and left for 10 min and a pink-red color change was considered positive.

3.4.3.6. Urease test

Urease test was used to determine the bacteria 's ability to produce urease and convert urea into CO_2, H_2O, and NH_4. After 18-24h pure colony was streaked on urea agar slants prepared using urea agar base (g/l) (Dextrose 1; Disodium phosphate 1.2; Monopotassium phosphate 0.8; NaCl 5; Peptone 1; Phenol red 0.012 and Agar 15) with addition of 40% (w/v) urea which was added to the agar base after sterilization and cooling down. The culture was incubated 35-37ºC for up to 5 days. The presence of urease is indicated by color change from yellow to pink.

3.4.3.7. Lactose fermentation test

All the isolates were inoculated on Mac-conkey agar plates and incubated for 18 h at 37°C. The plates were then observed for growth coloration, a pinkish coloration of colonies as a positive reaction.

3.4.4. Identification using MALDI_TOF MS

Species level identification was done by MALDI-TOF MS using the standard protocol (Bruker Daltonics, Germany). After overnight growth on Nutrient agar, isolated colonies of the bacteria were used as target sample for MALDI-TOF. All the bacterial samples were processed as per the manufacturer's instructions. The Micro flex LT MALDI-TOF MS instrument was used for identification process. In the sample preparation process, isolated bacterial colony of freshly grown culture was picked with the tip of sterile wooden toothpick and a uniform smear was prepared onto a steel biotarget plate in duplicates. The sample was air dried for around 10 min. After air drying, 1.0 µl of matrix solution of -cyano-4-hydroxycinnamic acid (HCCA) was transferred over the dried uniform bacterial film. Again, 1.0 µl of formic acid (100%) was transferred on another replicate for on-plate extraction method. The sample was air dried at room temperature before inserting the analyzing plate into the MALDI. Finally, the sample was inserted into the MALDI and the results were collected (Gautam *et al.*, 2017).

3.4.5. Biofilm Assay

Biofilm formation was assessed using the crystal violet (CV) method, as described by Stepanović *et al*., (2007) with minor modification. Briefly, 3 ml of the diluted bacterial culture was taken and inoculated in Luria Bertani (LB) broth and incubated for 48h at 37°C in a test tube. After incubation, the biofilm was washed twice with distilled water to remove free floating planktonic bacteria and air-dried. The test tubes were stained with 0.1% CV for 40min at room temperature and washed three times with distilled water to remove excess dye. The remaining CV was solubilized by incubating with 95% ethanol for 20min under orbital shaking at room temperature. Using similar procedures, a negative control without any treatment (only LB) and a positive control with *E. coli* was set for comparison to quantify the adherent cells. The absorbance of the CV solution was then measured by UV spectrophotometer at OD_{570}. Biofilm production was classified as negative, weak, moderate, and strong based on the cutoff value, calculated according to the following formula, using the optical density (OD_{570}) values:

$$ODcutoff = ODavg\ of\ negative\ control + (3 * SD\ of\ ODs\ of\ negative\ control)$$

$$New\ OD = Mean\ OD - ODcutoff$$

The used criteria were as follows:

(i) OD ≤ ODcutoff = non-biofilm former

(ii) ODcutoff < OD ≤ 2×ODcutoff = weak biofilm former

(iii) 2×ODcutoff < OD ≤ 4×ODcutoff = Moderate biofilm former

(iv) 4×ODcutoff = strong biofilm former

All assays were performed in triplicate (Stepanović *et al*., 2007).

3.4.6. Antimicrobial Susceptibility Test

The susceptibility against antimicrobial agents was investigated by the Kirby-Bauer disc diffusion method according to the CLSI (2017) guidelines. The bacterial isolates were spread evenly across the surface of a MHA plate using a sterile swab, creating a lawn of growth. Antibiotic-impregnated paper disks, each containing a specific concentration of antibiotics, were placed on the agar surface, using forceps to dispense each antimicrobial disk one at a time. The plates were incubated at 37°C for 24 hours, after which the zones of inhibition around the disks were measured in millimeters (Hudzicki, 2012). These measurements were compared to standardized guidelines from the CLSI 2017 to classify the bacteria as susceptible, intermediate, or resistant to each antibiotic tested, providing essential information for clinical treatment decisions. The following 7 selected antibiotics: ampicillin (10µg), cefepime (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), gentamycin (10µg), cephalexin (µg), and chloramphenicol (30 µg) were used for investigation because these are the antibiotics that represent different classes of antibiotics that are commonly used to treat infections caused by *Enterobacter* Spp. (Delgado Valverde *et al*., 2013).

Table 1 CLSI (2017) guidelines

Antibiotics	S	I	R
Ciprofloxacin	≥17	14-16	≤13
Cefepime	≥25	-	≤18
Gentamicin	≥15	13-14	≤12
Ceftriaxone	≥31	21-30	≤20
Cephalexin	≥23	20-22	≤19
Amoxicillin	≥13	-	≤12
Chloramphenicol	≥18	13-17	≤12

3.4.7. PCR amplification of 16S-23S rRNA intergenic spacer regions and 16S rRNA

3.4.7.1. Bacterial DNA extraction

For bacterial DNA extraction, 200 µl of lysis buffer and proteinase K were added to 200 µl of bacterial cells suspended in phosphate-buffered saline (PBS). The mixture was incubated at 56°C for 24 hours. Following this incubation, 200 µl of 96% ethanol was added to the supernatant, adjusting the final pH to 8.0. The solution was then transferred to a mini spin column tube, where it was washed with wash buffers AW1 and AW2 respectively to remove any excess ethanol. Finally, 50 µl of elution buffer was added to the filter and incubated at 56°C for 10 minutes. The sample containing the extracted DNA was diluted 100x, and 5 ml of the diluted extract was used for PCR (Woo *et al.*, 2001).

3.4.7.2. PCR amplification

DNase I-treated distilled water and PCR master mix (which contains deoxynucleoside triphosphates (dNTPs), PCR buffer, and Taq polymerase) was used in all PCR reactions. The PCR mixture (50 ml) has contained bacterial DNA, PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2 and 0.01% gelatin), 200 mM of each dNTPs and 1.0 U Taq polymerase. The bacterial DNA extract and control was amplified with 0.5 mM primers. The IGS region between 16S and 23S rRNA was amplified using FGPS1490' (TGC GGC TGG ATC ACC TCC TT) and FGPS132' (CCG GGT TTC CCC ATT CGG) primers (Laguerre et al., 1996). For the 16SrRNA, the primer pair rD1 (AAGGAGGTGATCCAGCC) and fD1 (AGAGTTTGATCCTGGCTCAG) (Weisburg et al., 1991), were used to generate a 1500bp PCR product. The PCR condition was a 95ºC initial denaturation for 5 minutes followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 30 seconds with a final extension at 72ºC for 5 minutes. The same primers were then used to sequence the PCR product on the ABI 3730XL genetic analyzer (Table 1).

Table 2. Primers, primer sequences, and cycling conditions used in the 16S rDNA and 16S–23S ITS PCR reactions.

Assay	Primer name	Primer sequence	Annealing temperature (Ta)	Cycling conditions
16S rRNA PCR	rD1'	AAGGAGGTGATCCAGCC	55°C	95°C→5min; 35 cycles of 95°C→30s, 55°C→1min, 72°C→30s, and 72°C→5min
	fD1'	AGAGTTTGATCCTGGCTCAG		
16S-23S IGS PCR	FGPS1490'	TGC GGC TGG ATC ACC TCC TT	55°C	
	FGPS132'	CCG GGT TTC CCC ATT CGG		

3.4.7.3. Gel electrophoresis, Visualization, and Sequencing

Each amplified product was electrophoresed in 1.5% (w/v) agarose gel, with a molecular marker in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 volts for 1.5 hours. The gel was stained with ethidium bromide (0.5 mg/mL) for 15 minutes, then it was rinsed and photographed under ultraviolet light illumination. The PCR product of 16S rRNA was gel-purified using the QIAquick PCR purification kit (QIAgen, Hilden, Germany). The purified PCR product was sequenced. Both strands of the PCR product was sequenced twice with an ABI 3770XL automated sequencer according to manufacturers' instructions using the PCR primers rD1 and fD1 (Weisburg *et al*., 1991).

3.5. Data Analysis

Experiments were conducted in triplicate or as specified. Phenotypic characterization data were analyzed using descriptive statistics and are presented in figures and tables. Additionally, Microsoft excel and OriginPro8.5 statistical software were utilized to generate graphical representations depicting the effects of pH, temperature, and salt concentration on the bacterial strain.

The 16s rRNA gene sequences were compared to sequences in the public database using the Basic Local Alignment Search Tool (BLAST, Zhang *et al*., 2000) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). The gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. This comparison was performed through multiple

sequence alignment using the MEGA 11 program, as described by (Tamura *et al.*, 2021). The phylogenetic tree was constructed by maximum likelihood method (Tamura *et al*., 2021).

3.6. Ethical Clearance

Ethical clearance for the study was obtained from the Ethical Review Committee of Adama Science and Technology University (Ref. No. RECSoANS/BIO/09/22; August 29, 2022). Prior to accessing and analyzing the data, all the bacterial cultures were completely anonymized, removing any identifying information.

CHAPTER 4

4. RESULTS

4.1. Isolation of *Enterobacter* species from environment and clinical samples

Enterobacter spp. was successfully isolated from both soil and clinical samples. During the isolation process, a variety of distinct colony types were observed, each differing in color and shape, which indicates the diversity of the bacterial population (Figure 5).

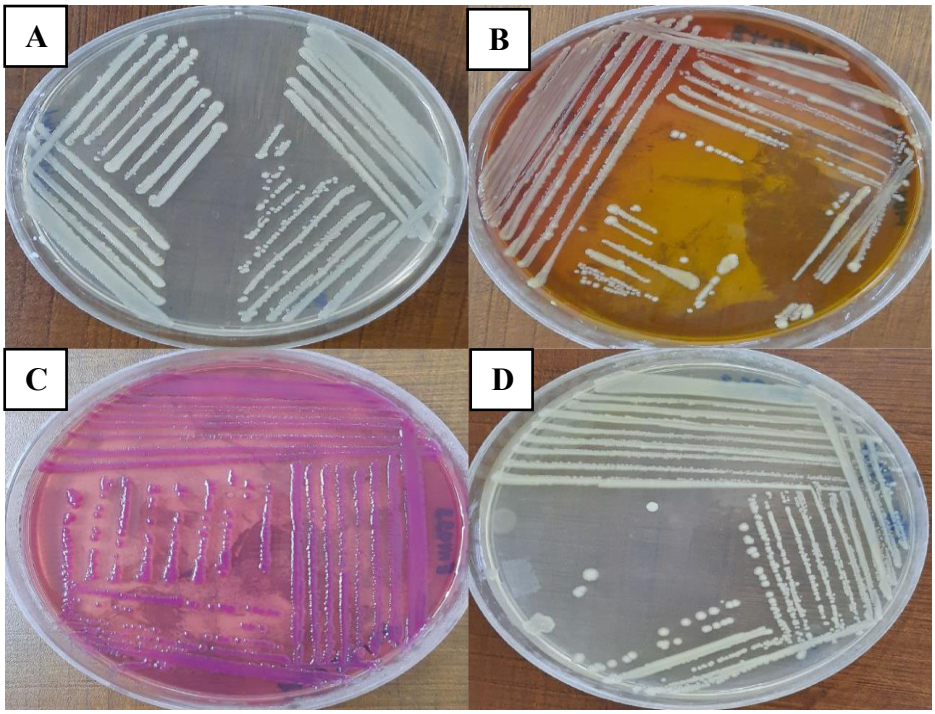

Figure 5. ENV053 grown on different media at 37^0C: MHA (A), LA (B), MAC (C), and NA (D)

A total, 54 pure bacterial colonies were isolated from the environmental samples, while 34 pure bacterial colonies were obtained from the clinical samples, following a series of consecutive re-streaking procedures. This systematic approach ensured the purity and integrity of the isolated colonies, providing a solid foundation for further characterization and analysis of the *Enterobacter* spp. present in these different environments.

4.2. Biochemical and Morphological Characterization of Bacterial Isolates

In this study, a total of 88 bacterial isolates were identified, comprising 54 from environmental samples and 34 from clinical samples. Among the 54 soil isolates, 35 (64.8%) were gram-negative, while 19 (35.2%) were gram-positive. In the clinical samples, 23 out of 34 isolates (67.65%) were confirmed as gram-negative, and 11 (32.35%) were gram-positive.

Biochemical testing revealed that 21 (60%) of the environmental isolates and 20 (90.9%) of the clinical isolates were identified as *Enterobacter* spp. All 21 environmental isolates tested positive for catalase, citrate utilization, Voges-Proskauer (VP), motility, and glucose fermentation. They were negative for the oxidase, methyl red (MR), and indole tests, with variable results for the urease test (Table 2). As shown in Figure 5 above, *Enterobacter* spp. exhibited smooth, creamy rods with a mucoid texture and no spore formation. Similarly, the 20 clinical isolates tested positive for citrate utilization, catalase, motility, and glucose fermentation, while they were negative for oxidase, indole, and urease tests.

Table 3. Biochemical characteristics of *Enterobacter* spp. isolated from environmental and clinical samples

	Biochemical Characteristics											
ISOLATE CODE	Shape	Gram-stain	Catalase	Indole	Citrate	Urease	MR	VP	Motility	H_2S	Lactose	Glucose
A010	R	-	+	-	+	-	-	+	+	-	+	+
A017	R	-	+	-	+	-	-	+	+	-	+	+
AR006	R	-	+	-	+	+	-	+	+	-	+	+
AY006	R	-	+	-	+	+	-	+	+	-	+	+
B004	R	-	+	-	+	+	-	+	+	-	+	+
B010	R	-	+	-	+	+	-	+	+	-	+	+
B017	R	-	+	-	+	+	-	+	+	-	+	+
IPS01	R	-	+	-	+	+	-	+	+	-	+	+
ENV048	R	-	+	-	+	+	-	+	+	-	+	+
ENV049	R	-	+	-	+	+	-	+	+	-	+	+
ENV052	R	-	+	-	+	+	-	+	+	-	+	+
ENV053	R	-	+	-	+	+	-	+	+	-	+	+
ENV056	R	-	+	-	+	+	-	+	+	-	+	+
ENV057	R	-	+	-	+	+	-	+	+	-	+	+
ENV058	R	-	+	-	+	+	-	+	+	-	+	+
ENV059	R	-	+	-	+	+	-	+	+	-	+	+
ENV060	R	-	+	-	+	+	-	+	+	-	+	+
ENV061	R	-	+	-	+	+	-	+	+	-	+	+
ENV062	R	-	+	-	+	+	-	+	+	-	+	+
ENV063	R	-	+	-	+	+	-	+	+	-	+	+
ENV064	R	-	+	-	+	+	-	+	+	-	+	+
CL15	R	-	+	-	+	+	-	+	+	-	+	+
CL16	R	-	+	-	+	+	-	+	+	-	+	+
CL17	R	-	+	-	+	+	-	+	+	-	+	+
CL18	R	-	+	-	+	+	-	+	+	-	+	+
CL19	R	-	+	-	+	+	-	+	+	-	+	+
CL20	R	-	+	-	+	+	-	+	+	-	+	+
CL21	R	-	+	-	+	+	-	+	+	-	+	+
CL22	R	-	+	-	+	+	-	+	+	-	+	+
CL23	R	-	+	-	+	+	-	+	+	-	+	+
CL24	R	-	+	-	+	+	-	+	+	-	+	+
CL25	R	-	+	-	+	+	-	+	+	-	+	+
CL26	R	-	+	-	+	+	-	+	+	-	+	+
CL27	R	-	+	-	+	+	-	+	+	-	+	+
CL28	R	-	+	-	+	+	-	+	+	-	+	+
CL29	R	-	+	-	+	+	-	+	+	-	+	+
CL30	R	-	+	-	+	+	-	+	+	-	+	+
CL31	R	-	+	-	+	+	-	+	+	-	+	+
CL32	R	-	+	-	+	+	-	+	+	-	+	+

CL33	R	-	+	-	+	+	-	+	+	-	+	+
CL34	R	-	+	-	+	+	-	+	+	-	+	+

Key: +: positive for the result, -: negative for the result, MR: methyl red, R: rod shaped, VP: Voges–Proskauer

4.3. Identification using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Out of 54 environmental bacterial isolates characterized using MALDI-TOF MS, 21 (38.9%) were identified as *Enterobacter* spp., 13 (24.1%) belonged to the *Bacillus* genus, 6 (11.1%) were *Priestia megaterium*, 2 (3.7%) were *Klebsiella pneumoniae*, and 5 (9.26%) were from the *Pseudomonas* genus. The remaining 7 isolates (12.96%) could not be identified. Notably, *Enterobacter* spp. were the most prevalent isolates recovered from the environmental soil samples. Among the 21 *Enterobacter* isolates, five distinct species were identified: 9 (42.9%) as *Enterobacter cloacae*, 6 (28.6%) as *Enterobacter bugandensis,* 3 (14.3%) as *Enterobacter ludwigii,* 3 (14.3%) as *Enterobacter asburiae*. *E. cloacae* was the most abundant species, representing the majority of *Enterobacter* isolates recovered from the environmental samples. *E. bugandensis* was the second most prevalent species in the environment (Table 3).

Out of 34 clinical bacterial isolates, 20 (58.82%) were *Enterobacter* spp., 7 (20.58%) were *Staphylococcus* spp., 2 (5.88%) were *Klebsiella pneumoniae*, 4 (11.76%) were *Bacillus pumillus*, and 1 (2.94%) was from *Exiguobacterium* spp. Among the *Enterobacter* isolates, two distinct species were identified: 13 (65%) were *E. cloacae* and 7 (35%) were *E. aerogenes* (see Table 3).

Table 4. MALDI-TOF MS Identification of environmental and clinical bacteria

Genus's level	Species level	Code of isolates	Number of isolates
Soil samples			
Bacillus	*B. cereus*	C017, IPS02, IPS03, ENV041, 47, 54, 55	7
	B. pumilus	ENV042, 43, 44	3
	B. thuringiensis	B008, Q001	2
	B. stabekisii	A001	1
Enterobacter	*E. asburiae*	B004, ENV048, ENV053	3
	E. bugandensis	A017, B010, B017, IPS01, ENV049,52	6
	E. ludwigii	A010, AR006, AY006	3
	E. cloacae	ENV056, 59,60,62,63,64	6
	E. aerogenes	ENV057, 58, ENV61	3
Pseudomonas	*P. monteilii*	B002	1
	P. putida	A002, D005, AY010, BY010	4
Priestia	*P. megaterium*	A009, A011, C005, Q003, ENV045,46	6
Klebsiella	*K. pneumonia*	ENV050,51	2
Clinical samples			
Enterobacter	*E. cloacae*	CL31-34,17-20, 25-29	13
	E. aerogenes	CL15,16,21-24, 30	7
Staphylococcus	*S. xylosus*	CL01-04,07-09	7
Bacillus	*B. pumillus*	CL10-13	4
Klebsiella	*K. pneumonia*	CL05 & CL06	2
Exiguobacterium	*E. aurantiacum*	CL14	1

4.4. Biofilm Assay Result

In the current study, all isolates identified as *Enterobacter* spp. through MALDI-TOF MS were evaluated for their biofilm formation ability. The mean optical density at 570 nm (OD_{570}) was calculated for each isolate to classify them as either strong or weak biofilm producers. The results indicated that almost all the isolates from environmental samples were strong biofilm producers. As shown in Figure 6, the lowest OD_{570} value recorded among the

environmental isolates was 0.5587 for isolate AR006, while the highest was 1.033 for isolate ENV053. Four isolates AR006, AY006, B004, and B010 were found to be moderate biofilm producers based on the cut value stated on Stepanović *et al*., (2007). According to the criteria established by Stepanovic *et al*., the optical density cutoff (ODc) value for the negative control in the environmental isolates was determined to be 0.145. Eighty percent 17 (80%) of the environmental isolates exhibited optical density values that were more than four times of the ODc value, categorizing them as strong biofilm producers.

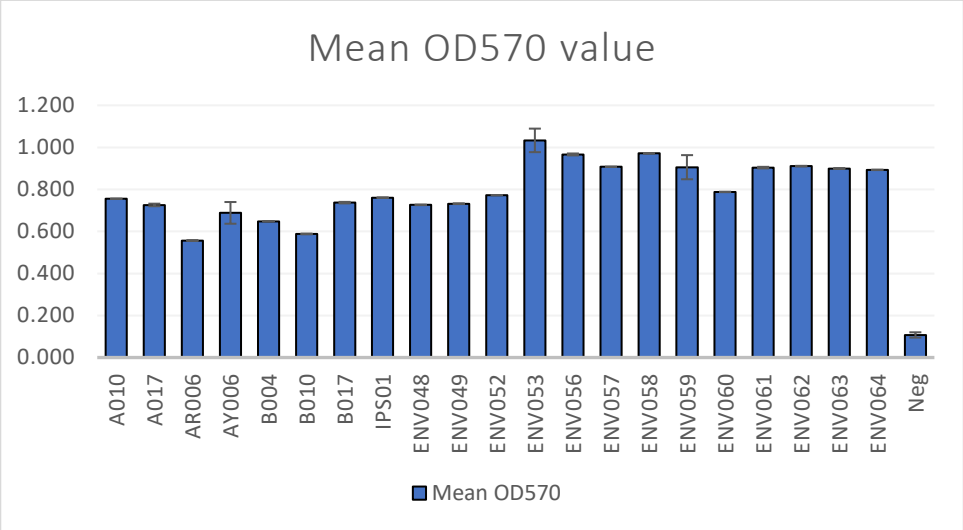

Figure 6. Mean OD570 Value of isolates: soil *Enterobacter species*

In contrast, the clinical isolates exhibited a minimum OD570 value of 0.74 (CL16) and a maximum of 1.11 (CL26). Isolates CL15 and CL16 were classified as moderate biofilm producers (Figure 7). Furthermore, 18 (90%) of the clinical isolates surpassed four times the ODc value of the negative control, indicating that they are also strong biofilm producers.

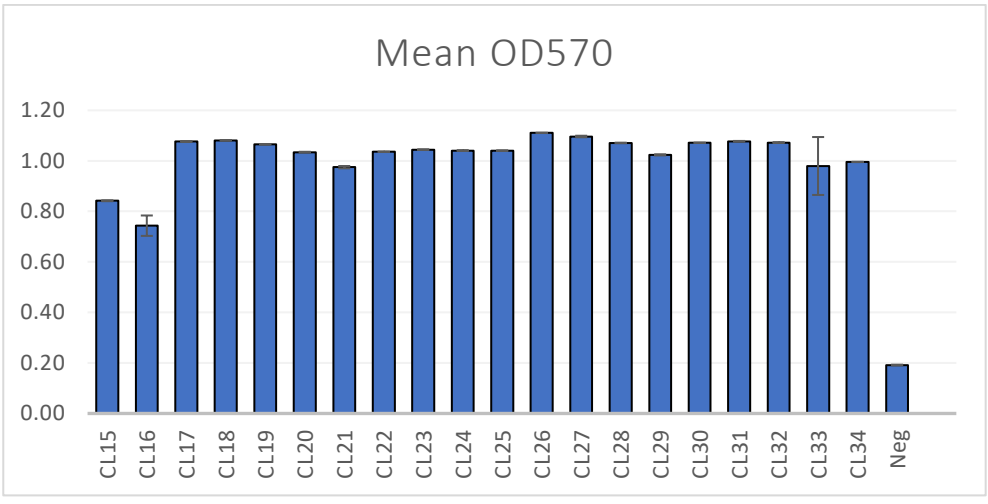

Figure 7. Mean OD570 Value of isolates: clinical *Enterobacter* species.

4.5. Antimicrobial Susceptibility Test Result

In this study, antimicrobial resistance in *Enterobacter* spp. was assessed against a range of antibiotics representing key drug classes commonly used to treat *Enterobacter* infections, including cephalosporins, aminoglycosides, and fluoroquinolones.

For the environmental isolates, the antimicrobial resistance profiles of 21 identified *Enterobacter* spp. were investigated using the disk diffusion method. The highest levels of resistance were observed for chloramphenicol 21 (100%) and ampicillin 20 (95.23%) followed by cephalexin 18 (85.71%). Moderate resistance was noted for ceftriaxone 12 (57.14%) and gentamicin 12 (57.14%), while lower levels of resistance were recorded for cefepime 6 (28.6%) and ciprofloxacin 9 (42.85%). Additionally, Table 4 shows that some *Enterobacter* isolates exhibited intermediate susceptibility to certain antibiotics, including cefepime 8 (38.09%), and ceftriaxone 8 (38.09%), ciprofloxacin 5 (23.8%), and cephalexin 3 (14.28).

For the clinical isolates, 20 isolates were examined for their antibiotic resistance profile. High levels of resistance were observed for ampicillin 19 (95%), chloramphenicol 17 (85%), and cephalexin 16 (80%). Lower level of resistance was noted for cefepime 7 (35%). Notably, the isolates from the clinical setting demonstrated high susceptibility to cefepime 12 (60%). Furthermore, some *Enterobacter* isolates exhibited intermediate susceptibility to certain antibiotics, including gentamicin 5 (25%) and ciprofloxacin 4 (20%), highlighting the complex and varied resistance patterns within these samples.

Table 5. Percentage of antibiotic resistance of *Enterobacter* spp. isolated from environmental and clinical samples

Class of Antibiotics	**Name of Antibiotics**	**Number (%) of Resistance, Intermediate, and Susceptibility Of Isolates (N=21 soil & N=20 clinical)**					
		R		**I**		**S**	
		Soil	**Clinic**	**Soil**	**Clinic**	**Soil**	**Clinic**
Fluoroquinolones	Ciprofloxacin	9 (42.85)	13 (65)	5 (23.8)	4 (20)	7 (33.33)	3 (15)
Aminoglycosides	Gentamycin	12 (57.14)	14 (70)	0 (0)	5 (25)	9 (42.85)	1(5)
Cephalosporins (1^{st},3^{rd},	Cefepime	6 (28.6)	7 (35)	8 (38.09)	1 (5)	7 (33.33)	12 (60)
and 4^{th} generation)	Ceftriaxone	12 (57.14)	14 (70)	8 (38.09)	0 (0)	1 (4.76)	6 (30)
	Cephalexin	18 (85.71)	16 (80)	3 (14.28)	3 (15)	0 (0)	1 (5)
Beta lactams	Ampicillin	20 (95.23)	19 (95)	0 (0)	1 (5)	1 (4.76)	0 (0)
Phenicol	Chloramphenicol	21 (100)	17 (85)	0 (0)	2 (15)	0 (0)	0 (0)
Mean		66.67	71.43	16.32	12.14	17.0	16.43

Key: R: resistance, I: intermediate, S: susceptible

4.7. Molecular detection of Intergenic spacer and 16S rRNA region

The IGS regions provide a powerful method for distinguishing between phylogenetically closely related species. In this study, six bacterial isolates, represented in lanes 2 to 7, were selected for further molecular analysis based on their identification through MALDI-TOF MS. These isolates underwent PCR amplification, resulting in a segment length of 400 bp. As illustrated in Figure 8, isolates ENV048 and ENV052 exhibited similar DNA banding patterns following gel electrophoresis. The comparable band positions suggest that the IGS regions of these samples are genetically similar, indicating that they may belong to the same species or closely related species. This finding complements the MALDI-TOF MS results, which identified ENV048 and ENV052 as *E. bugandensis*. In contrast, the remaining isolates, ENV049, ENV053, ENV061, and ENV064, displayed distinct DNA banding patterns, suggesting genetic diversity among these isolates. This observation supports the notion that they may belong to different species or strains, as indicated by the MALDI-TOF MS results which designated ENV053, 61, and 64 as *E.asburiae*, *E,aurogenes*, and *E.cloacae*, respectively.

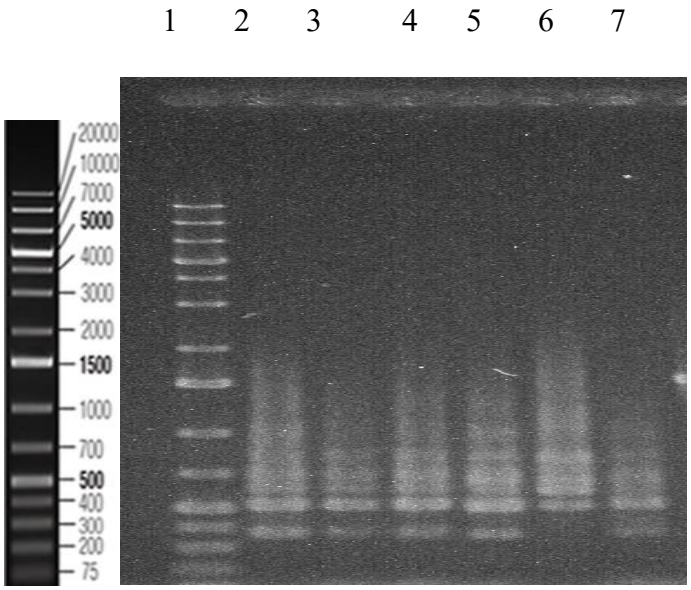

Figure 8. Agarose gel electrophoresis showing IGS Region

Key: Lane 1- ladder, Lane 2- ENV048 FGPL vs FGPS, Lane 3- ENV049 FGPL vs FGPS, Lane 4- ENV052 FGPL vs FGPS, Lane 5- ENV053 FGPL vs FGPS, Lane 6- ENV061 FGPL vs FGPS, and Lane 7- ENV064 FGPL vs FGPS

4.8. 16S rRNA sequencing and Phylogenetic tree Analysis for ENV053

Two isolates, ENV053 and CL15, were purposively selected for 16S rRNA sequencing based on their clinical significance, drug resistance profiles, and recent global reports indicating that these isolates are emerging carbapenemase producers (Marchetti et al., 2024). Although the sequencing results for CL15 were unsuccessful, the 16S rRNA sequencing for ENV053 yielded positive results. The analysis indicated that this isolate belongs to *E. asburiae*, exhibiting a high sequence similarity of 98%. The next closest similarity was observed with *E. ludwigii*, showing 97% similarity with another *Enterobacter* species. These sequence results also corroborate the MALDI-TOF MS findings. As shown in Figure 9, the phylogenetic neighbor is MK968090.1, *Enterobacter* sp. strain IAUK3001.

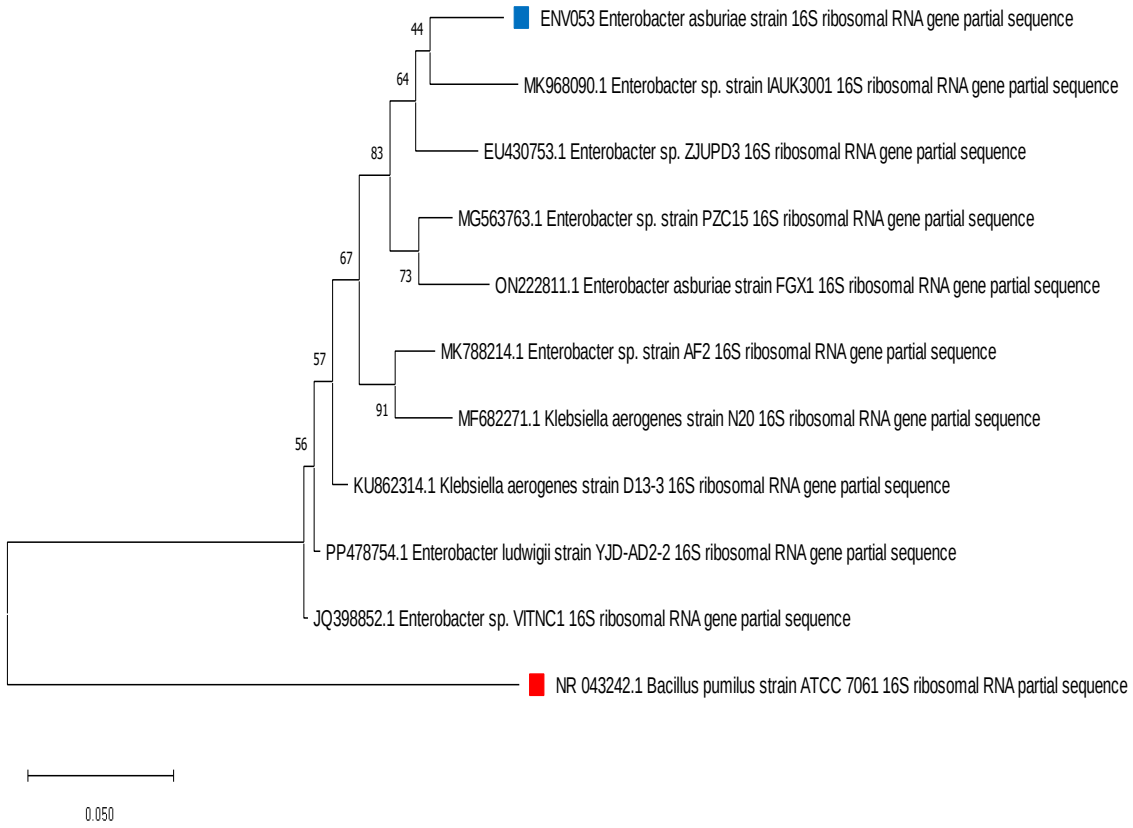

Figure 9. Molecular Phylogenetic analysis by Maximum Likelihood method (Tamura & Nei, 1993).

CHAPTER 5

5. DISCUSSION

In this study, a total of 41 (21 environmental and 20 clinical) isolates were identified as *Enterobacter* Spp. The study revealed that the isolates identified as *Enterobacter* spp. were gram negative bacteria with rod shape. The biochemical tests revealed all the identified *Enterobacter* Spp. were positive for citrate, catalase, VP, motility, and glucose fermentation tests. Our findings corroborate with the recent finding by Al-Muhana and colleagues., (2023) which highlighted this biochemical testing methods represent some of the most effective approaches for differentiating *Enterobacter* spp. from other related microbial species. The biochemical assays utilized in this study were found to be highly reliable and discriminatory tools for this purpose (Al-Muhana *et al*., 2023).

To achieve more definitive identification and confirmation of isolated *Enterobacter* strains, MALDI-TOF MS was employed as an additional technique. In this study, the MALDI-TOF results in the identification of 21 isolates from soil samples as *Enterobacter* spp., while only 7 isolates could not be identified using this technique. Additionally, 20 isolates from clinical settings were also identified as *Enterobacter* spp. These advanced proteomic methods have emerged as powerful complementary tools that enhance confidence in bacterial classification beyond what traditional biochemical assays can provide (Franco-Duarte *et al*., 2019). Previous research, including the work by Singhal *et al*., (2015) has shown that MALDI-based identification is highly accurate, with rare instances of misidentification of microorganisms. MALDI-TOF MS has gained significant attention for its routine application in bacterial identification, and studies have demonstrated its beneficial use in various environmental research contexts (Ashfaq *et al*., 2022).

Bacteria typically produce biofilms as an adaptive response to environmental stressors or to overcome therapeutic pressures. The present study revealed that over 17 (80%) of soil samples and 18 (90%) of clinical samples demonstrated strong biofilm production, indicating that *Enterobacter* spp. isolates have the potential to adapt to both environmental and clinical settings. Notably, clinical isolates exhibited slightly higher levels of biofilm formation compared to environmental isolates. This finding is consistent with studies by Karami *et al*., (2020) which demonstrated a higher prevalence of strong biofilm producers among clinical isolates of

Pseudomonas aeruginosa compared to environmental ones. This suggests that, on average, clinical isolates are more proficient biofilm producers than their environmental counterparts. Study by (Abebe, 2020; Folliero *et al*., 2021) underscores the potential risks for treatment and management strategies, as the enhanced ability to form biofilms may increase the resilience of these bacteria against antimicrobial agents in various settings. The enhanced ability to form biofilms may increase the resistance of *Enterobacter* spp. to antimicrobial agents. This necessitates ongoing surveillance of resistance patterns and the development of new antibiotics or adjunct therapies that can effectively target biofilm-associated infections.

Based on our study, the antimicrobial susceptibility testing of *Enterobacter* isolates from soil and clinical samples revealed significant resistance patterns, with both sources showing high resistance to chloramphenicol, ampicillin, and cephalexin. According to the findings by Davin-Regli & Pagès, (2015), *E. cloacae* exhibits an inherent resistance to several classes of β-lactam antibiotics, including ampicillin and amoxicillin, first-generation cephalosporins, and cefoxitin. Notably, in the current study, cefepime emerged as a consistently effective treatment option, demonstrating high susceptibility across both sample types. This aligns with previous findings by Siedner *et al*., (2014) that suggests cefepime could be viable choices for treating *Enterobacter* infections. Ciprofloxacin could also be used as the second treatment option for *Enterobacter* infections. According to EFDA (2023) Ciprofloxacin indicated for treating lower respiratory tract infections in adult patients caused by *Escherichia coli, Klebsiella pneumoniae, and Enterobacter* spp.

The high levels of resistance observed in both environmental and clinical isolates present a significant challenge in treating Enterobacter infections. The resistance seen in Enterobacter species, particularly those isolated from soil samples, indicates an increased risk of transmission, leading to infections that are difficult to treat. The increasing abundance of antibiotic resistance genes (ARGs) in soil has significant implications for human health and clinical practice. This is primarily due to the connection between soil and food production, which creates a direct pathway for novel resistance determinants to transfer from soil to food and subsequently to human commensal and pathogenic bacteria (Zhu et al., 2019). This situation contributes to more severe illnesses and higher mortality rates. These bacteria can easily enter the human population through contaminated water, food products, or direct contact with soil, potentially resulting in

widespread outbreaks. It is important to note that the de novo emergence of bacterial resistance can originate from a single bacterium and subsequently disseminate globally. A notable example of this is the mcr-1 gene, which confers resistance to colistin. This gene spread following a single de novo mutation event that likely occurred in China, driven by the extensive use of colistin in swine farms (Liliana Serweci´nska, 2020). Because of the bacterial widespread outbreaks, the effectiveness of existing antibiotics may diminish, complicating treatment options and increasing healthcare costs as reliance grows on more expensive or toxic alternatives. The dominance of β-lactam antibiotics such us ampicillin resistance highlights the potential for treatment failures in clinical settings. The results underscore the urgency for healthcare providers to consider resistance patterns when prescribing antibiotics.

The study also involved six bacterial isolates identified through MALDI-TOF MS, which were further analyzed using PCR to amplify the IGS region between the 16S and 23S rRNA genes. Based on a study by Maeda *et al*., (2000) IGS regions could help for distinguishing closely related bacteria solely based on the size separation of PCR products in electrophoresis. The results indicated that isolates ENV048 and ENV052 shared a high degree of genetic similarity, while the other four isolates displayed notable differences in their genetic profiles. This variation suggests evolutionary divergence among the *Enterobacte*r spp. isolates, indicating that those with similar DNA profiles may belong to the same strain, whereas distinct patterns could signify different strains. In a study by Poaceae, (2017), the intergenic spacer region, particularly the segment adjacent to 26S rDNA, was utilized as a molecular marker, providing a significant opportunity for the phylogenetic reconstruction of Stipa.

The PCR amplification of the 16S rRNA region using rD1 and fD1 primers confirmed the DNA extracted from ENV053 was indeed of bacterial origin. Subsequent sequencing of the 16S rRNA gene identified the isolate as *E. asburiae*, consistent with the earlier MALDI-TOF MS identification. A BLAST search in NCBI revealed that the sequence exhibited a 97% similarity with the type strain of *E. asburiae* and other *Enterobacter* spp., indicating close genetic relationships. In one study, comparisons of 16S rRNA sequences revealed that the type strain of *E. cloacae* exhibited a high similarity of 97.8% with other *Enterobacter* spp. when compared to reference strains in GenBank (Hadi *et al*., 2023). Another study by Woo *et al*., (2001) also showed the relatedness of the studied isolate to strain *E. cloacae* based on 16S rRNA gene

sequencing. Based on the findings, 16S rRNA sequencing is a highly effective method for accurately identifying bacterial isolates. The resulting phylogenetic tree also showed that the isolate is closely related to *E. ludwigii* and other *Enterobacter* spp. Findings from Lehner *et al*., (2004) indicated that through phylogenetic analysis using16S rRNA gene sequence data, they were able to determine the lineage of the bacterial strain and identify if there is a second phylogenetically distinct lineage within the *E. sakazakii* species they studied.

6. CONCLUSION AND RECOMMENDATION

Conclusion

Our study indicated that *Enterobacter* spp. are prevalent in both environmental and clinical samples. The isolates from both settings exhibited high biofilm formation and significant resistance to common therapeutic options in the clinical setting, including chloramphenicol, ampicillin, cephalexin, and ceftriaxone. Our study also showed that cefepime demonstrated promising activity against *Enterobacter* from both sources, indicating its potential as an effective treatment option. Molecular characterization using 16S rRNA sequencing confirmed the strain-level identification of isolate ENV053 as belonging to *Enterobacter* spp., which is consistent with the results from MALDI-TOF MS and biochemical tests.

Recommendation

- Larger-scale study by collecting *Enterobacter* isolates from diverse environmental and clinical settings across a wider geographical area. This will provide a more comprehensive understanding of the of the prevalence of *Enterobacter* spp. as well as the magnitude of antimicrobial resistance patterns and help identify any regional variations.
- To examine in-depth molecular and biochemical analyses to characterize the specific resistance mechanisms employed by the *Enterobacter* isolates, such as the production of beta-lactamases, efflux pumps, or target modifications. This knowledge can inform the design of novel antimicrobial agents or the repurposing of existing ones.
- To perform whole-genome sequencing analysis of the *Enterobacter* isolates to identify the genetic determinants responsible for antimicrobial resistance. This information can help elucidate the underlying mechanisms of resistance and guide the development of targeted interventions.
- To launch campaigns to raise awareness about antimicrobial resistance and the role of biofilms in persistent *Enterobacter* infections among the public and healthcare stakeholders. Additionally, promote education on the proper use of antibiotics, emphasizing the importance of responsible disposal practices to prevent environmental contamination. This includes informing the public not to dispose unused or expired antibiotics in soil or water sources, as such actions can contribute to the development of resistant bacterial strains.

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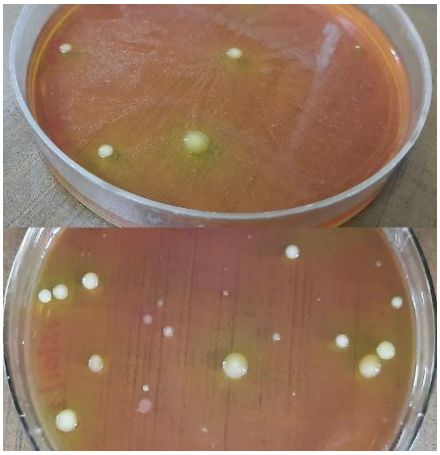
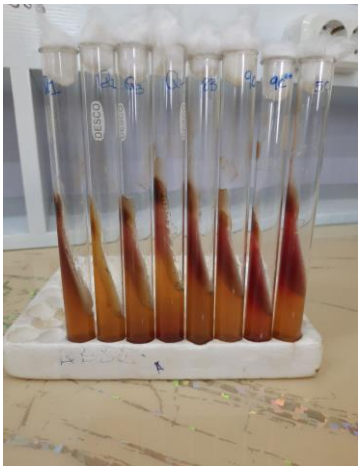
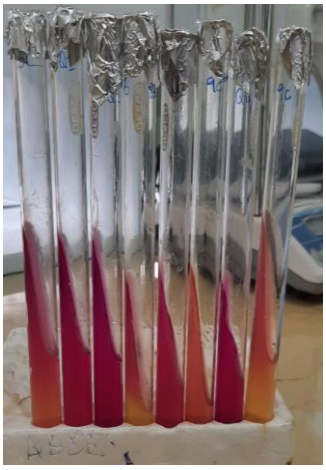
Appendices

Appendix A. Illustrative pictures during sample collection and processing

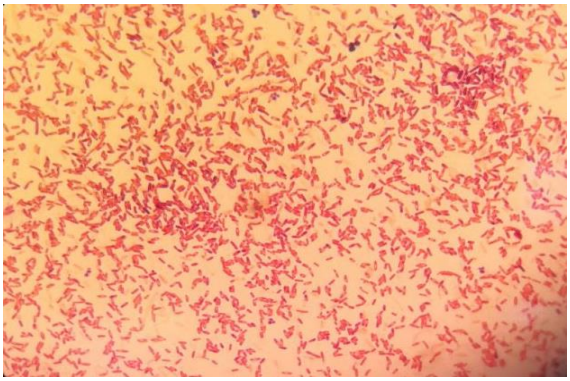
Sample collection from soil

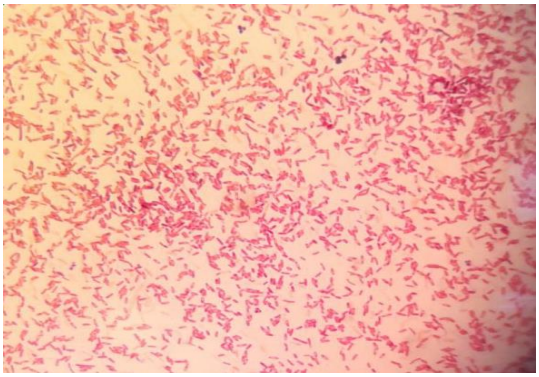






Primary Isolates of *Enterobacter* spp. (A) and some selected biochemical tests against *Enterobacter* species (B)



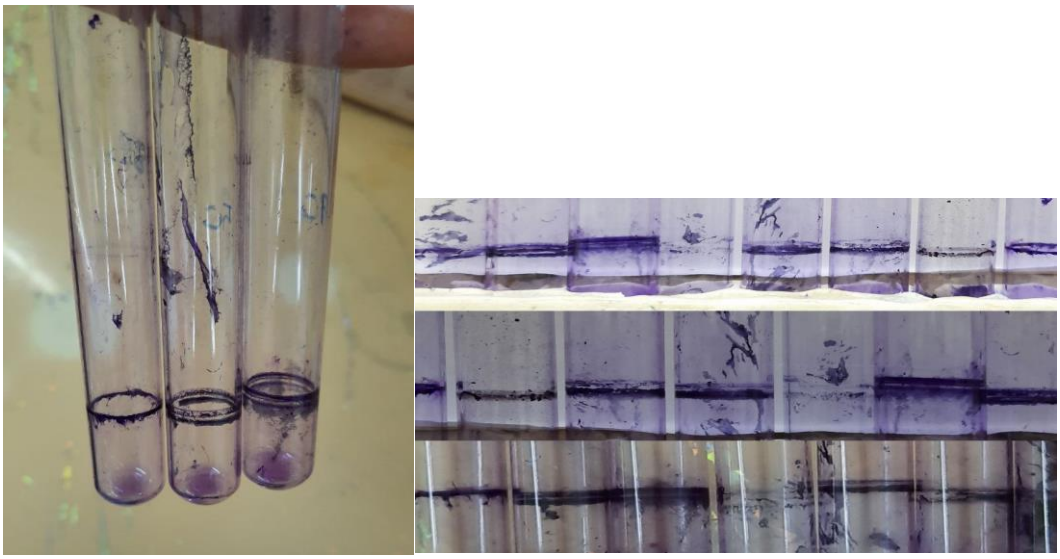


Gram staining results of isolates from soil and clinical setting

Appendix B. Biofilm Mean OD result for the *Enterobacter* spp. isolated from Soil and Health facility respectively, at 48h

Code of isolates	OD_{570} value of each bacterial isolate				
	Rep. 1	Rep.2	Rep.3	Mean OD	STD
A010	0.755	0.755	0.756	**0.755**	0.00058
A017	0.723	0.733	0.722	**0.726**	0.00608
AR006	0.557	0.556	0.557	**0.557**	0.00058
AY006	0.748	0.659	0.658	**0.688**	0.05168
B004	0.648	0.648	0.649	**0.648**	0.00058
B010	0.586	0.589	0.589	**0.588**	0.00173
B017	0.738	0.733	0.74	**0.737**	0.00361
IPS01	0.759	0.762	0.761	**0.761**	0.00153
ENV048	0.727	0.725	0.727	**0.726**	0.00115
ENV049	0.733	0.733	0.73	**0.732**	0.00173
ENV052	0.772	0.773	0.773	**0.773**	0.00058
ENV053	1.001	1.098	1.001	**1.033**	0.05600
ENV056	0.969	0.969	0.961	**0.966**	0.00462
ENV057	0.907	0.908	0.907	**0.907**	0.00058
ENV058	0.971	0.972	0.972	**0.972**	0.00058
ENV059	0.972	0.872	0.873	**0.906**	0.05745
ENV060	0.789	0.788	0.787	**0.788**	0.00100
ENV061	0.908	0.901	0.902	**0.904**	0.00379
ENV062	0.909	0.911	0.912	**0.911**	0.00153
ENV063	0.899	0.899	0.9	**0.899**	0.00058
ENV064	0.892	0.893	0.894	**0.893**	0.00100
NEG	0.122	0.1	0.1	**0.107**	0.01270
CL15	0.841	0.844	0.841	**0.84**	0.00173
CL16	0.72	0.72	0.79	**0.74**	0.04041
CL17	1.076	1.077	1.076	**1.08**	0.00058
CL18	1.08	1.081	1.082	**1.08**	0.00100
CL19	1.065	1.065	1.066	**1.07**	0.00058
CL20	1.036	1.034	1.033	**1.03**	0.00153
CL21	0.97	0.979	0.976	**0.98**	0.00458
CL22	1.036	1.035	1.036	**1.04**	0.00058
CL23	1.044	1.044	1.045	**1.04**	0.00058
CL24	1.04	1.041	1.041	**1.04**	0.00058
CL25	1.04	1.041	1.04	**1.04**	0.00058
CL26	1.11	1.111	1.111	**1.11**	0.00058

CL27	1.097	1.099	1.093	**1.10**	0.00306
CL28	1.07	1.071	1.072	**1.07**	0.00100
CL29	1.021	1.025	1.025	**1.02**	0.00231
CL30	1.071	1.071	1.072	**1.07**	0.00058
CL31	1.077	1.079	1.076	**1.08**	0.00153
CL32	1.072	1.072	1.073	**1.07**	0.00058
CL33	0.913	0.914	1.112	**0.98**	0.11461
CL34	0.996	0.996	0.995	**1.00**	0.00058
NEG	0.192	0.19	0.193	**0.19**	0.00153



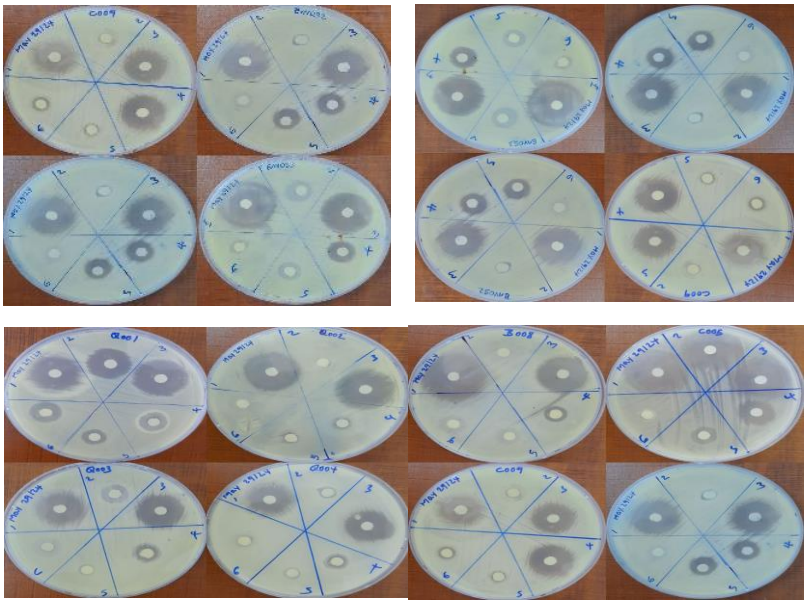
Qualitative observation of biofilm formation for selected bacteria after 48h, at 37°C

Appendix C. AST result for the *Enterobacter* spp. isolated from Soil and clinical samples respectively

Code of bacterial isolates	Based on their Diameter of zone of inhibition (mm)							MALDI-TOF Identification
	Ciprofloxacin	**Gentamicin**	**Cefepime**	**Ceftriaxone**	**Cephalexin**	**Amoxicillin**	**Chloramphenicol**	
Disk content	**5 µg**	**30 µg**	**10 µg**	**30 µg**	**30 µg**	**10 µg**	**30 µg**	
A010	I	R	I	R	R	R	R	*E. ludwigii*
A017	I	S	S	R	R	S	R	*E. ludwigii*
AR006	S	S	S	R	R	R	R	*E. bugandensis*
AY006	S	R	I	R	R	R	R	*E. bugandensis*
B004	I	R	I	I	R	R	R	*E. bugandensis*
B010	S	R	S	I	R	S	R	*E. asburiae*
B017	S	R	I	R	R	R	R	*E. bugandensis*
IPS01	S	R	I	I	R	R	R	*E. ludwigii*
ENV048	S	R	R	I	R	R	R	*E. asburiae*
ENV049	S	S	R	I	R	R	R	*E. bugandensis*
ENV052	I	S	R	I	R	R	R	*E. bugandensis*
ENV053	I	R	R	I	R	R	R	*E. asburiae*
ENV056	R	S	I	R	R	R	R	*E. cloacae*
ENV057	R	S	R	R	R	R	R	*E. cloacae*
ENV058	R	S	R	R	I	R	R	*E. cloacae*
ENV059	R	R	S	R	R	R	R	*E. cloacae*
ENV060	R	S	S	R	I	R	R	*E. cloacae*
ENV061	R	R	S	I	R	R	R	*E. cloacae*
ENV062	R	S	I	S	R	R	R	*E. cloacae*
ENV063	R	R	I	R	R	R	R	*E. cloacae*
ENV064	R	R	S	R	I	R	R	*E. cloacae*
CL15	S	I	S	S	R	R	R	*E. aerogenes*
CL16	R	I	I	R	R	R	R	*E. aerogenes*
CL17	R	R	R	R	R	R	R	*E. cloacae*
CL18	R	R	S	R	R	R	R	*E. cloacae*
CL19	R	R	S	R	R	R	R	*E. cloacae*
CL20	R	R	R	R	R	R	R	*E. cloacae*
CL21	R	I	S	S	R	R	R	*E. aerogenes*
CL22	R	R	R	R	R	R	R	*E. aerogenes*
CL23	R	R	R	R	R	R	R	*E. aerogenes*
CL24	I	S	S	S	I	R	R	*E. aerogenes*

CL25	I	I	S	S	I	R	R	*E. cloacae*
CL26	I	R	R	R	R	R	R	*E. cloacae*
CL27	S	I	S	S	I	R	R	*E. cloacae*
CL28	R	R	R	R	R	R	R	*E. cloacae*
CL29	R	R	S	R	R	R	R	*E. cloacae*
CL30	I	R	S	R	R	R	I	*E. aerogenes*
CL31	R	R	S	R	R	R	I	*E. cloacae*
CL32	R	R	S	R	R	R	R	*E. cloacae*
CL33	R	R	R	R	R	I	R	*E. cloacae*
CL34	S	R	S	S	S	R	I	*E. cloacae*

Antibiotics	**S**	**I**	**R**
Ciprofloxacin	≥17	14-16	≤13
Cefepime	≥25	-	≤18
Gentamicin	≥15	13-14	≤12
Ceftriaxone	≥31	21-30	≤20
Cephalexin	≥23	20-22	≤19
Amoxicillin	≥13	-	≤12
Chloramphenicol	≥18	13-17	≤12

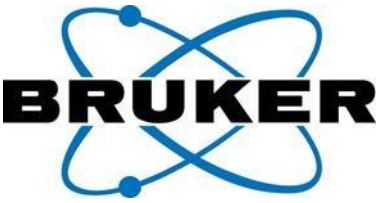


***Enterobacter* Spp.** tested for different types of antibiotics and Zone of disk size interpretation chart according to CLSI (2017) guidelines

Appendix D. MALDI-TOF MS identification result

Bruker MALDI Biotyper

Identification Results



Report Info:

Created by:	MBT-WIN10-LTSC\tof-user
Creation Date/Time:	2/5/2024 11:49 AM
Tests coverage:	30 of 30 Tests selected for this report

Run Info:

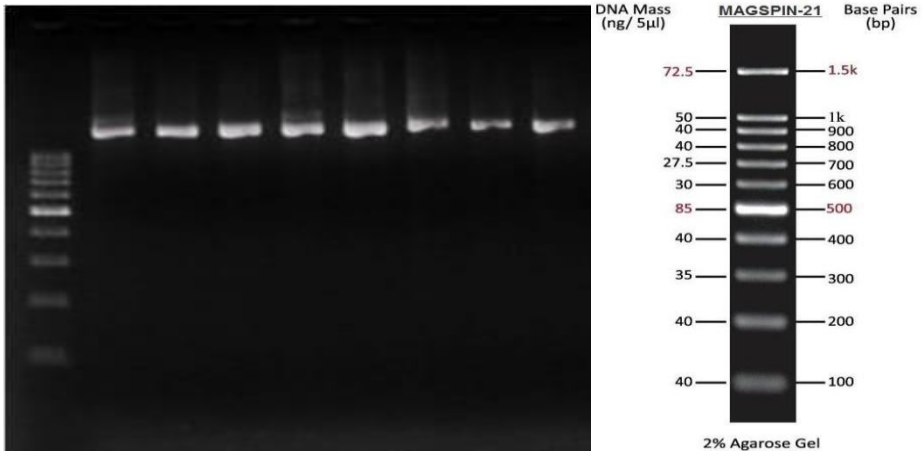
Run Identifier:	240205-1140-1011006065
Comment:	Abera and Tilaye ACB
Created by:	MBT-WIN10-LTSC\tof-user
Run Creation Date/Time:	2/5/2024 11:44 AM not yet
Run Result Review:	reviewed not yet approved
Run Result Approval:	30
Number of Tests:	Not yet performed
Validation:	
Validation Position:	1865142.70648
Instrument Identifier:	5.1.300
Server Version:	- / -
BTS / Matrix Lot Numbers:	

Result Overview

Example of Result overview table by MALDI-TOF MS				
Sample identifier (Type)	Sample Name	Organism (best match) Organism (second best match)	log(score)	Consistency
A017 (Sample)		Enterobacter bugandensis Enterobacter ludwigii	1.86 (+) 1.86 (+)	(B)
ENV053 (Sample)		Enterobacter asburiae Enterobacter asburiae	**2.12 (+++)** 2.1(+)	(A)
B010 (Sample)		Enterobacter bugandensis Enterobacter bugandensis	**2.01 (+++)** 1.98 (+)	(A)
IPS03 (Sample)		Bacillus cereus Bacillus cereus	1.99 (+) 1.99(+)	(A)
B017 (Sample)		Enterobacter bugandensis Enterobacter bugandensis	2 .15(+++) 2.14(+++)	(A)
A009 (Sample)		Priestia megaterium Priestia megaterium	**2.04 (+++)** 1.92 (+)	(A)
B008 (Sample)		Enterobacter ludwigii Enterobacter ludwigii	2.09 (+++) 2.07 (+++)	(B)
A014 (Sample)		No peaks found No peaks found	0 (-) 0 (-)	

Range	Interpretation	Symbols	Color
2.00 - 3.00	High Confidence Identification	(+++)	Green
1.70 - 1.99	Low Confidence Identification	(+)	Yellow
0.00 – 1.69	No organism Identification possible	(-)	Red

Appendix E. 16S rRNA data: Sanger Seq Chromatogram, NCBI Blast, Graphic summary



> ENV053

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Sequences producing significant alignments:

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter asburiae strain Ichip_C_2-10* 16S ribosomal RNA gene, partial sequence	Enterobacter asburiae	1290	1290	100%	0.0	100.00%	1439	MW487460.1
Enterobacter sp. ZJUPD3 16S ribosomal RNA gene, partial sequence	Enterobacter sp. ZJUPD3	1107	1107	100%	0.0	95.28%	1447	EU430753.1
Bacterium strain ZX05 16S ribosomal RNA gene, partial sequence	bacterium	1105	1105	100%	0.0	95.27%	1418	ON387320.1
Bacterium strain SZ515 16S ribosomal RNA gene, partial sequence	bacterium	1099	1099	100%	0.0	94.70%	1430	ON387161.1
Bacterium strain HB17-2 16S ribosomal RNA gene, partial sequence	bacterium	1096	1096	100%	0.0	94.99%	1408	ON387007.1
Enterobacteriaceae bacterium 124G 16S ribosomal RNA gene, partial sequence	Enterobacteriaceae bacterium 124G	1090	1090	100%	0.0	94.85%	1373	KM021070.1
Enterobacteriaceae bacterium strain Atelim3F1B 16S ribosomal RNA gene, partial sequence	Enterobacteriaceae bacterium	1086	1086	100%	0.0	94.70%	1392	MT386305.1
Enterobacter mori strain ADY18 16S ribosomal RNA gene, partial sequence	Enterobacter mori	1085	1085	100%	0.0	94.71%	1352	MH084804.1
Enterobacter sp. strain PZC15 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1083	1083	100%	0.0	94.70%	1414	MG563763.1
Enterobacter sp. SEB006 16S ribosomal RNA gene, partial sequence	Enterobacter sp. SEB006	1079	1079	100%	0.0	94.56%	1379	KJ027531.1
Klebsiella aerogenes strain SR9 16S ribosomal RNA gene, partial sequence	Klebsiella aerogenes	1079	1079	100%	0.0	94.56%	1477	MW959075.1
Enterobacteriaceae bacterium strain Atelim3F2 16S ribosomal RNA gene, partial sequence	Enterobacteriaceae bacterium	1074	1074	100%	0.0	94.13%	1402	MT386306.1
Leclercia adecarboxylata strain D114_CV2R 16S ribosomal RNA gene, partial sequence	Leclercia adecarboxylata	1074	1074	100%	0.0	94.41%	1444	MK883208.1
Pantoea rwandensis strain A11 16S ribosomal RNA gene, partial sequence	Pantoea rwandensis	1074	1074	100%	0.0	94.41%	1392	OQ916409.1
Enterobacter sp. AK2 16S ribosomal RNA gene, partial sequence	Enterobacter sp. AK2	1068	1068	100%	0.0	94.27%	1368	KR780041.1
Enterobacter sp. strain AF2 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1062	1062	100%	0.0	94.13%	1435	MK788214.1
Enterobacter sp. strain IAUK3001 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1062	1062	100%	0.0	94.13%	1430	MK968090.1
Uncultured bacterium clone 201406P19 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1057	1057	100%	0.0	93.98%	1441	KT280494.1
Enterobacter sp. strain PA102 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1057	1057	100%	0.0	93.98%	1384	OP739345.1